Video Article

Fluorescence and Bioluminescence Imaging of Subcellular Ca^{2+} in Aged Hippocampal Neurons.

María Calvo-Rodríguez[1], Carlos Villalobos[1], Lucía Nuñez[1]

[1]Instituto de Biologìa y Genética Molecular, Consejo Superior de Investigaciones Cientificas y Universidad de Valladolid

[2]Departamento de Bioquìmica y Biologìa Molecular y Fisiologìa, Universidad de Valladolid

Correspondence to: Carlos Villalobos at carlosv@ibgm.uva.es

URL: http://www.jove.com/video/53330
DOI: doi:10.3791/53330

Keywords: Excitotoxicity, NMDAR, cytosolic calcium, mitochondrial calcium, hippocampal neurons.

Date Published: 10/15/2015

Citation: Calvo-Rodríguez, M., Villalobos, C., Nuñez, L. Fluorescence and Bioluminescence Imaging of Subcellular Ca^{2+} in Aged Hippocampal Neurons.. *J. Vis. Exp.* (), e53330, doi:10.3791/53330 (2015).

Abstract

Susceptibility to neuron cell death associated to neurodegeneration and ischemia are exceedingly increased in the aged brain but mechanisms responsible are badly known. Excitotoxicity, a process believed to contribute to neuron damage induced by both insults, is mediated by activation of glutamate receptors that promotes Ca^{2+} influx and mitochondrial Ca^{2+} overload. A substantial change in intracellular Ca^{2+} homeostasis or remodeling of intracellular Ca^{2+} homeostasis may favor neuron damage in old neurons. For investigating Ca^{2+} remodeling in aging we have used live cell imaging in long-term cultures of rat hippocampal neurons that resemble in some aspects aged neurons in vivo. For this end, hippocampal cells are, in first place, freshly dispersed from new born rat hippocampi and plated on poli-D-lysine coated, glass coverslips. Then cultures are kept in controlled media for several days or several weeks for investigating young and old neurons, respectively. Second, cultured neurons are loaded with fura2 and subjected to measurements of cytosolic Ca^{2+} concentration using digital fluorescence ratio imaging. Third, cultured neurons are transfected with plasmids expressing a tandem of low-affinity aequorin and GFP targeted to mitochondria. After 24 h, aequorin inside cells is reconstituted with coelenterazine and neurons are subjected to bioluminescence imaging for monitoring of mitochondrial Ca^{2+} concentration. This three-step procedure allows the monitoring of cytosolic and mitochondrial Ca^{2+} responses to relevant stimuli as for example the glutamate receptor agonist NMDA and compare whether these and other responses are influenced by aging. This procedure may yield new insights as to how aging influence cytosolic and mitochondrial Ca^{2+} responses to selected stimuli as well as the testing of selected drugs aimed at preventing neuron cell death in age-related diseases.

Video Link

The video component of this article can be found at http://www.jove.com/video/53330/

Introduction

Excitotoxicity is one of the most important mechanisms contributing to neuronal damage and cell death in neurological insults such as ischemia, and in some neurodegenerative diseases such as Alzheimer's disease [1]. This type of neurotoxicity is mainly mediated by glutamate acting on Ca^{2+}-permeable, ionotropic NMDA receptors (NMDAR) [2]. Exposure of cultured neurons to glutamate can lead to excitotoxicity [3], which causes neuronal apoptosis [4]. We and others have previously reported that neuronal vulnerability to NMDA-induced apoptosis may change with development *in vitro* and aging [5-8].

It is widely accepted that an increase in the cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) leads to cells activation. However, if this rise is too high and/or sustained enough, it can trigger cell death [9]. Moreover, it has been proposed that excitotoxicity requires mitochondrial Ca^{2+} uptake [10], since treating neurons with a mitochondrial uncoupler protected neurons against glutamate-induced cell death [11]. If mitochondria take up too much Ca^{2+}, the opening of the mitochondrial permeability transition pore may occur, leading to release of cytochrome c and other pro-apoptotic factors, and inducing apoptosis. We have recently shown that this mitochondrial Ca^{2+} uptake is directly related to the age-dependant susceptibility to excitotoxicity, by directly measuring NMDA-induced mitochondrial Ca^{2+} uptake in single hippocampal neurons [5], a method which is reported in this article. The hippocampus, involved in physiological processes such as learning, memory and other cognitive processes [12], is highly vulnerable to aging and neurodegenerative disorders [13]. It has been proposed that, after several weeks in vitro, cultured hippocampal neurons show a number of typical characteristics of aged neurons [14]. Accordingly, long-term cultured hippocampal neurons may provide a comprehensive model to investigate Ca^{2+}-mediated mechanisms of enhanced excitotoxicity in aging.

The overall goal of the method presented is, therefore, to investigate substantial changes in intracellular Ca^{2+} homeostasis or Ca^{2+} remodeling in the aging brain including the differential Ca^{2+} responses elicited by NMDA receptor agonists in a long-term cultured hippocampal neurons. The method includes a detailed description of the culture of rat hippocampal neurons and the monitoring of cytosolic and mitochondrial Ca^{2+} concentrations by fluorescence and bioluminescence imaging in individual neurons, respectively. Fluorescence imaging of cytosolic Ca^{2+} in cultured neurons is a standard procedure. However, this method is less reliable for subcellular Ca^{2+} measurements including mitochondrial Ca^{2+}. Reasons for this include lack of proper targeting of synthetic probes and inappropriate affinity for Ca^{2+} concentrations that may change in

mitochondria from the low µM level even to the mM level. The use of Ca^{2+} probes based on proteins as for instance aequorin, has allowed the targeting to subcellular organelles and the use of derivatives different Ca^{2+} affinities using different coelenterazines or mutated probes lacking specific Ca^{2+} binding sites [15]. In this way, bioluminescence imaging of cells expressing mitochondria-targeted aequorin may allow the monitoring of mitochondrial Ca^{2+} concentrations in individual neurons. Yet, this procedure may require the use of photon counting cameras or ultrasensitive CCD cameras for bioluminescence imaging [16-18]. This method may yield novel results that should be confirmed in more established brain aging models as, for instance, brain slices from old animals.

Protocol

Ethics Statement: Procedures involving animal subjects have been handled under protocols approved by the Valladolid University animal housing facility in agreement with the European Convention 123/Council of Europe and Directive 86/609/EEC.

1. Short and Long-term culture of rat hippocampal neurons

1. **Preparation of poly-D-lysine coated, 12 mm glass coverslips.**
    1. Sterilize 12 mm diameter glass coverslips in ethanol for at least 24 h. Allow them to dry under sterile conditions.
    2. Distribute the coverslips on a strip of parafilm in a Petri dish. Cover the surface of each coverslip with 200 µl of 1 mg/ml poly-D-lysine. Allow treating overnight.
    3. The next day wash the coverslips with double distilled sterile water every 15 minutes for 90 min under sterile conditions.
    4. Fill a four-well multidish plate with 500 µl of Neurobasal Culture Medium per well. Neurobasal Culture Medium should be supplemented with 10 % fetal bovine serum, 2 % B27, 1 µg/ml gentamicin and 2 mM L-glutamine.
    5. Place the coverslips in the multidish plate. Maintain them in a humidified 37 ºC and 5 % CO_2 incubator until use.

2. **Isolation of hippocampal neurons from neonatal rats.**
    1. Prepare 1.8 ml papain solution (directly purchased) in a vial (20 u./ml) under sterile conditions: To obtain a final concentration of 20 u./ml add around 40 µl papain in 1.8 ml Hank's plus 0.6% BSA (µl should be calculated depending on the supplier conditions). Hank's 0.6% BSA is prepared by mixing 85 ml of HBSS medium with 15 ml of 4% bovine serum albumin (BSA, prepared solving 4 g of BSA in 100 ml HBSS). Prepare it in a sterile hood.
    2. Incubate the papain in Hank's plus 0.6% BSA for 30 min at 37 ºC before use. Filter the solution using a 0.22 µm filter prior to use.
    3. Prepare Ham's F-12 medium by adding Dulbecco's Modified Eagle's Medium powder (13.5 g per l) in 900 ml of double distilled sterile water. Then add 6 g HEPES and 336 mg $NaHCO_3$ and adjust the pH to 7.42 using NaOH 4N. Add sterile water in order to obtain a 1 l solution and filter it using a 0.20 µm polyethersulfone (PES) bottle top filter. Carry this process in a sterile hood. Finally saturate the solution with CO_2 under sterile conditions before use. Store solutions at 4 ºC and use them chilled.
    4. Euthanize newborn (P0) rat pups by decapitation and wash the head in sterile HBS medium. Open the skull with the help of sterile scissors. Extract the brain with a spatula and wash it quickly in Ham's F-12 medium.
    5. Make a diagonal cut with the aid of a scalpel in each hemisphere (Figure 1 B), and carry it to a Petri dish containing Ham's F-12 medium.
    6. Discard meninges carefully with the help of dissection forceps and under a magnifying glass.
    7. Identify the hippocampus in a characteristic concave location over the cortex using magnifying glass. Then, separate the hippocampus from the cortex by pulling carefully from one border and removing it from its position using eye scissors.
    8. Transfer the hippocampal tissue to a 4-well plate filled with sterile Hank's medium lacking Ca^{2+} and Mg^{2+} plus 0.6% BSA and wash hippocampal tissue. Without removing the media cut the tissue in small pieces (around 2x2 mm) using the same eye scissors.
    9. Transfer hippocampal pieces to a vial containing 1.8 ml pre-filtered papain solution. Then incubate them at 37 ºC with occasional, gentle shaking. 15 min later, add 90 µl of DNase I solution (50 µg/ml final) and incubate for 15 additional min.
    10. Transfer the tissue to a 10 ml centrifuge tube and wash the fragments with fresh Neurobasal Culture Medium. Obtain cell suspension by passing tissue fragments through a 5 ml plastic pipette.
    11. Centrifuge cell suspension at 160 x *g* for 5 min. Remove the supernatant using a plastic sterile Pasteur pipette and suspend pellet carefully with a 1 ml automated pipette in 1 ml of Neurobasal Culture Medium.
    12. Measure cell density using a Neubauer counting chamber. Put the glass coverslip over the Neubauer chamber. Add 10 µl of cell suspension. Make sure the cell suspension enters the chamber uniformly and making no bubbles. Count the number of cells under the microscope and perform corresponding calculations to obtain a suitable drop of 40 - 80 µl containing 30 x 10^3 cells. The total number of cells will depend on the number of animals used.

3. **Short-term and long-term culture of rat hippocampal neurons**.
    1. Over the four-well multidish plate containing 500 µl of Neurobasal Culture Medium prepared before and kept in the incubator, plate around 30 x 10^3 cells in a drop of about 50 µl to each well of the multidish plate containing one poly-D-lysine-coated, 12 mm diameter glass coverslip.
    2. Maintain primary hippocampal cells in a humidified 37 ºC and 5 % CO_2 incubator for 2-5 days in vitro (DIV, short-term, young cultures) or >15 DIV (Long-term, aged cultures) before experiments without changing the culture media.

2. Fluorescence Imaging of Cytosolic Ca^{2+} concentration

1. **Preparation of test solutions for fluorescence imaging.**
    1. Prepare an external HEPES-buffered saline (HBS) solution containing, in mM: NaCl 145, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, glucose 10, sodium-HEPES 10 (pH 7.42).

2. Prepare an external, Mg^{2+}-free, HEPES-buffered saline (HBS) solution containing, in mM: NaCl 146, KCl 5, $CaCl_2$ 1, glucose 10 and HEPES 10 (pH 7.42).
3. Solve NMDA in Mg^{2+}-free, HEPES-buffered saline (HBS) at a final concentration of 100 µM and supplement it with 10 µM glycine.
4. Prepare a HBS solution containing KCl instead of NaCl by solving (in mM): KCl 145, $MgCl_2$ 1, $CaCl_2$ 1, glucose 10 and HEPES 10 (pH 7.42).

2. **Loading of hippocampal cells with fluorescent calcium probe fura2/AM.**
   1. Take the cell culture containing coverslips off the incubator and wash them with HBS medium at room temperature by transferring them to a new four-well multidish plate containing 500 µl of HBS per well.
   2. Incubate cells with fura2/AM 4 µM (prepared in the same HBS medium) for 60 min at room temperature (25 ºC) in a dark place.
   3. After 60 min, wash coverslips with fresh HBS medium.

3. **Recording fluorescence images of cytosolic [Ca^{2+}] in cultured cells.**
   1. Turn on the lamp, microscope, perfusion system, fluorescence camera and computer.
   2. Place the coverslips in a thermostated platform for open 12 mm glass coverslips on the stage of the inverted microscope and select a microscopic field using a 40x objective (oil, NA:1.3). Perfuse cells continuously with pre-warmed (37 ºC) HBS medium in absence or presence of test substances. Maintain the flow at a rate of about 5-10 ml/min.
      Note: Cell perfusion system for living cells is mounted in a thermostated platform for open 12 mm glass coverslips. 8-lines gravity-driven perfusion system equipped with a valve controller is used to perfuse the solutions. A vacuum pump is responsible for removing any excess medium. Solutions are heated using an in-tube heating system.
   3. Capture a background image with the shutter closed at both excitation wavelengths.
   4. Epi-illuminate cells alternately at 340 and 380 nm. Record light emitted at 520 nm every 5-10 s with a fluorescence camera, which is filtered by a fura-2 dichroic mirror.
   5. When the recording period is finished, store the complete sequence of images emitted at 520 nm in the computer for further analysis.

4. **Analysis of recorded fluorescent images.**
   1. Open the experiment file. Using the aquacosmos software, click on 'Ratio' and select the desired ratio range. Calculate the pixel by pixel ratio in the resulting images in order to obtain a sequence of ratio images.
   2. Subtract background by adjusting the 'background elimination'. Press 'start calculation'.
   3. Press 'all times sequence' button and erase the ancient regions of interest (ROIs). For quantitative analysis of individual cells, establish new regions of interest or ROIs corresponding to individual neurons. Average all ratio values in each pixel corresponding to each ROI and each image to obtain a recording of ratio fluorescence values for individual ROIs (cells).
   4. Export ratio fluorescence values corresponding to each region of interest to a program for graphing the individual recordings. To do this, click on 'Graph'. Select 'Average' and 'use current ROI to all images'. Click on 'calculate' and save the 'txt.file'.
   5. Make the corresponding calculations for estimating the size of the rises in ratio fluorescences in response to each stimulation using a suitable data analysis and graphing software.

3. Bioluminescence Imaging of Mitochondrial Ca^{2+} concentration

1. **Transfection of cultured hippocampal neurons with the mitGAmut plasmid.**
   NOTE: Cells are first transfected with the mitGAmut plasmid containing a mitochondria-targeted sequence and a mutated aequorin lacking a Ca^{2+} binding site fused to the green fluorescent protein (GFP). This construction detects large rises in mitochondrial Ca^{2+} uptake and allows the identification of transfected cells by the GFP fluorescence. The plasmid was developed and kindly provided by P. Brûlet and co-workers [18] (CNRS, Paris).
   1. Prepare Neurobasal TF, containing Neurobasal Culture Medium, lacking fetal bovine serum, B27, gentamicin and 2 mM L-glutamine.
   2. Prepare 50 µl of Neurobasal TF plus 2.5 µl transfection reagent in a vial (Solution A). Prepare 50 µl of Neurobasal TF plus 4 µg mitGAmut plasmid in a vial (Solution B). Add gently solution B over solution A. Allow mixing during 20 min, without shaking.
   3. Transfer the coverslips containing hippocampal neurons to new multidish plates containing 200 µl of fresh Neurobasal TF.
   4. Add drop by drop 100 µl of solution A + B over every coverslip. Incubate for 30 min. Remove the medium. Wash once cells with fresh Neurobasal TF. Return the coverslips to the original Neurobasal Culture Medium.
   5. Culture neurons for 24 additional h at 37 ºC and 5% CO_2 after transfection to allow efficient expression and targeting of the probe.

2. **Preparation of test solutions for bioluminescence imaging.**
   1. Prepare HBS solutions containing NMDA 100 µM or 145 mM KCl as above (Step 2.1). Prepare HBS solution containing 10 mM Ca^{2+} plus digitonin 100 µM.

3. **Reconstitution of mitochondria-targeted aequorin with coelenterazine n.**
   1. Transfer coverslips containing transfected hippocampal neurons to a four-well plate containing 200 µl of HBS.
   2. Add 4 µl of coelenterazine n (200 µM) to 200 µl of HBS for a final concentration of 4 µM and mix gently. Incubate for 2 h at room temperature (25 ºC) and in the dark.

4. **Recording bioluminescence images of mitochondrial [Ca^{2+}].**
   1. Turn on the microscope, the lamp, the perfusion system, bioluminescence camera and the computer.
   2. Transfer reconstituted coverslips to a thermostated (37 ºC) perfusion chamber and perfuse continuously with pre-warmed HBS solution at a rate of 5-10 ml/min.
   3. Using a 40x objective (oil, NA: 1.3), select a microscopic field containing cells expressing the apoaequorins as shown by fluorescence emission associated with expression of green fluorescent protein (GFP) using the FITC filters. A typical field may contain 1-2 transfected cells. Capture the GFP fluorescence image, using a CCD camera.

4. Turn off the microscope light. Remove the dichroic-containing box located in the light pathway. Turn off the excitation light and close the dark-box for complete darkness.
5. Perfuse cells at 5-10 ml/min with HBS medium with or without test solutions pre-warmed at 37 ºC.
6. Capture photonic emission images every 10 seconds with a photon-counting camera handled with an image processor.
7. At the end of each experiment, permeabilize cells with 0.1 mM digitonin in 10 mM $CaCl_2$ in HBS in order to release all remaining photonic emissions.
   Note: These photonic emissions must be added up in order to estimate the total photonic emissions, a value required for calibration in Ca^{2+} concentrations.
8. Store bioluminescence images in the computer with the GFP associated fluorescence image captured before the photon counting imaging.

5. **Analysis of bioluminescence images reflecting mitochondrial [Ca^{2+}].**
   1. Quantify photonic emissions using specific software. Photonic emissions can be converted to mitochondrial free Ca^{2+} concentration ($[Ca^{2+}]_{mit}$) using the algorithm described by Brini et al. [15].
   2. Open the experiment and the GFP fluorescence image. Select regions of interest (ROIs) with the help of specific software by drawing the shape of the transfected cells according to the fluorescence images captured at the beginning of the experiment.
   3. Paste the same ROIs on every image of the sequence screen captures\mitochondrial 1.eps. Total photonic emissions in each ROI are computed with specific software to obtain the luminescence emission value (L) for each cell at each point in time.
   4. Add up all the photonic emissions in the bioluminescence images, including the ones obtained after digitonin permeabilization, using the specific software, to obtain a bioluminescence image containing all the photonic emissions.
   5. Export values of photonic emissions for every region of interest to a specific software for calculations. To do this, click on 'Graph' in order to compute the bioluminescence values for every ROI at each time. Select 'total value' and 'use current ROI to all images. Press 'calculate'. Save the 'txt.file' and export data to a worksheet. For every ROI, $[Ca^{2+}]_{mit}$ is calculated using the following algorithm:
      $Ca^{2+}](M) = [R + (R \cdot K_{TR}) - 1] / [K_R - (R \cdot K_R)]$;
      where,
      $R = [L/(L_{total}\lambda)]^{1/n}$
      L is the luminescence emitted at the time of measurement in each region of interest.
      L_{total} is the total luminescence remaining after addition of the counts present in the tissue at that time at the time of measurement for each region of interest. λ, K_{TR}, K_R and n are constants whose values depend on combination of aequorin (wild type or mutated) and coelenterazin used (wild type or n type) as well as the recording temperature [16]. For the combination used here (mutated AEQ and coelenterzine n), at 37 ºC, we used the following values: K_R=8,47x10^7, K_{TR}=156,6x10^3, n=1,204 and λ=0,138.

6. Make the corresponding calculations for estimating the size of the rises in bioluminescence in response to each stimulus using a suitable data analysis and graphing software.

Representative Results

Here we describe a simple method to assess Ca^{2+} remodeling and the effects of NMDA on cytosolic and mitochondrial [Ca^{2+}] in aged neurons. Fig. 1 shows the schematic of the procedure for isolating and culturing hippocampal neurons from neonatal rats. Before starting, we need to prepare sterile, poly-D-lysine coated, glass coverslips and locate them in a 4-well dish. Then, neonatal rats are killed and the brain removed. After isolating the hippocampus, tissue is carefully dispersed using papain. Isolated cells are washed and plated on coated coverslips. Then cells are cultured for 2-5 DIV or >15 DIV to get young or aged cultures, respectively, and used for Ca^{2+} imaging experiments.

Using the above strategy, it is possible to have young and old neurons from the same specimen to test, for example, whether NMDA induces differential effects on cytosolic [Ca^{2+}] in young cultures than in older cultures. Fig. 2 shows that NMDA 100 µM induces larger increases in cytosolic [Ca^{2+}] in older cultures than in young cultures. Likewise, NMDA induces cell death in old neurons but not in young neurons [5]. In cases where Ca^{2+} responses are very high, similar experiments can be carried out with Ca^{2+} probes with less affinity for Ca^{2+} to avoid dye saturation like, for instance fura4F [5]. In the same manner, it is also possible to learn whether resting levels of cytosolic [Ca^{2+}] are different. Moreover, the combination of this protocol with quantitative immunofluorescence using antibodies specific for NMDAR subunits may allow correlate changes in responsiveness with differences in expression of NMDA receptor subunits provided that specific antibodies are available [5]. Measurements of cytosolic [Ca^{2+}] can be used also for assessing other relevant parameters including Ca^{2+} store content, resting permeability to Ca^{2+}, Ca^{2+} clearance rates and their possible differences between young and aged neurons.

In a similar fashion, it is also possible to test the effects of such stimuli on mitochondrial [Ca^{2+}]. Fig. 3 shows an example of bioluminescence imaging of hippocampal neurons transfected with mitochondria-targeted aequorin. Release of photonic emissions after stimulation is a function of the rise in mitochondrial [Ca^{2+}] achieved. Notice that NMDA-induced rises in mitochondrial [Ca^{2+}] are much larger in aged neurons than in young hippocampal neurons, where NMDA barely increases photonic emissions. These results may contribute to explain why NMDA induces apoptosis only in aged neurons but not in young cultures of hippocampal cells [5]. In the same manner, it is possible to test for additional differences in mitochondrial Ca^{2+} handling between young and aged neurons using protocols specifically designed to test, for instance, Ca^{2+} exit from mitochondria, mitochondrial Ca^{2+} uptake in permeabilized neurons and mitochondrial Ca^{2+} uptake induced by Ca^{2+} release from intracellular stores. Moreover, this methodology could be used to test for drugs affecting mitochondrial Ca^{2+} overload that can be of interest for neuroprotection against excitotoxicity.

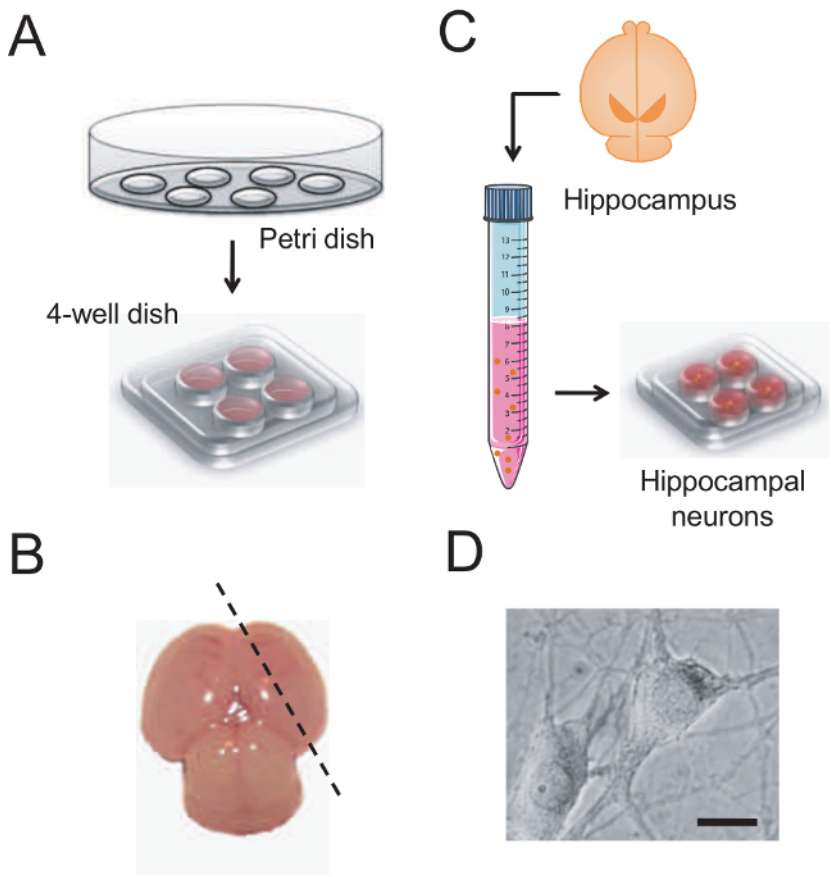

Figure 1: Procedure for isolation of primary rat hippocampal neurons. A. Poly-D-lysine, 12 mm glass coated coverslips are prepared in a Petri dish and finally transferred to a 4-well dish. **B.** Dorsal view of a rat brain. The blade indicates where the cut should be made. **C.** Obtaining a suspension of primary hippocampal neuron cells. After removing the hippocampus, a cell suspension is obtained. Then cells are plated in the 4-well dishes containing the poly-D-lysine coated coverslips. **D.** Bright field image showing primary rat hippocampal neurons in culture. Bar represents 10 µm.

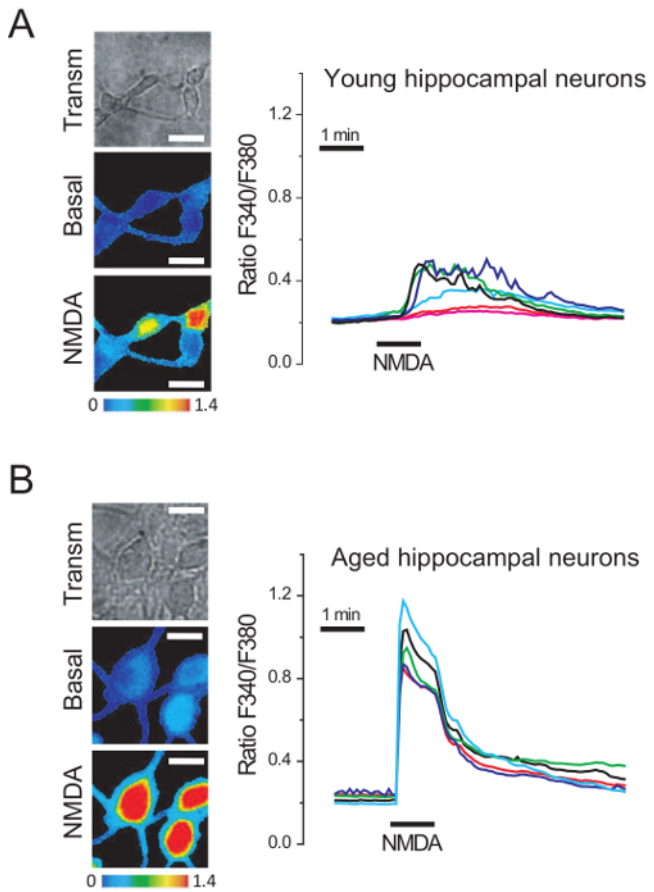

Figure 2: NMDA increases cytosolic [Ca^{2+}] in hippocampal neurons. Short- and long term cultured hippocampal neurons were loaded with fura2/AM and subjected to fluorescence imaging. Pictures show representative bright field and pseudocolor images (Ratio F340/F380) of short-term (**A**) and long-term (**B**)hippocampal neurons before (basal) and after stimulation with 100 µM NMDA. Warmer colors reflect elevated cytosolic [Ca^{2+}] (pseudocolor scales are shown at the bottom). Traces show representative, single-cell recordings of cytosolic [Ca^{2+}] in response to 100 µM NMDA in short-term (**A**) and long-term (**B**) cultured hippocampal neurons. Note that cytosolic [Ca^{2+}] increases are much larger in long-term than in short-term cultured neurons. Bar represents 10 µm.

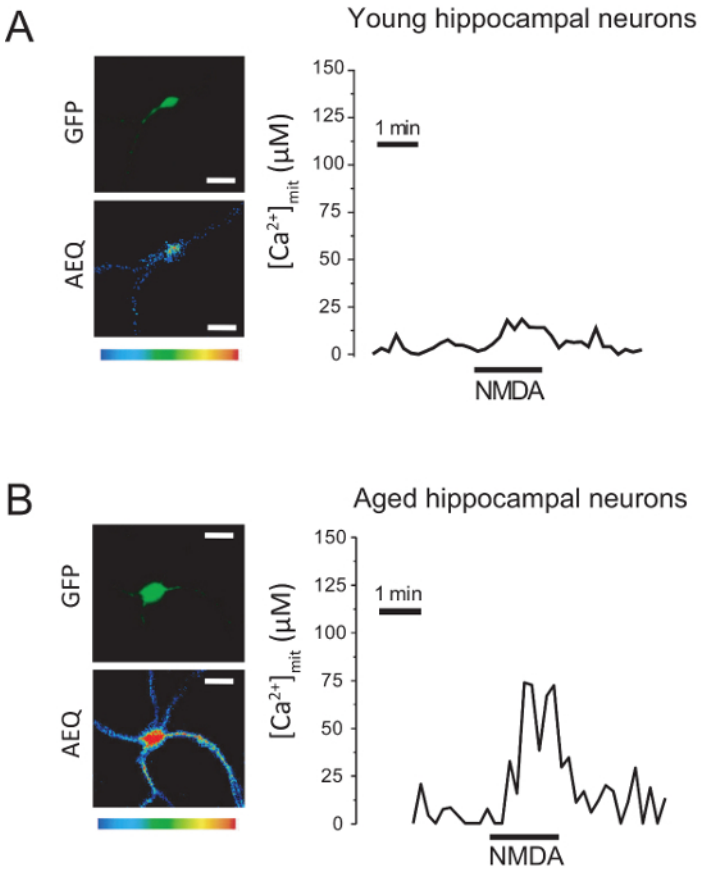

Figure 3: NMDA induces mitochondrial Ca^{2+} overload in hippocampal neurons. Cultured hippocampal neurons were transfected with the low-affinity, mitochondria targeted aequorin fused to GFP, incubated with 4 µM coelenterazine and subjected to bioluminescence imaging of mitochondrial [Ca^{2+}]. Pictures show the fluorescence (GFP) and accumulated photonic emissions (aequorin bioluminescence) images of representative short- (**A**) and long-term (**B**) cultured hippocampal neurons. Luminescence intensity is coded in pseudocolor (1 to 16 photonic emissions per pixel). Recordings show the release of photonic emissions (expressed as mitochondrial [Ca^{2+}]) in short- (**A**) and long-term (**B**) cultured hippocampal neurons. NMDA 100 µM increased mitochondrial [Ca^{2+}] in aged neurons but not in young cultured hippocampal neurons. Bar represents 10 µm.

Discussion

The remodeling of intracellular Ca^{2+} homeostasis in the aging brain has been related to cognitive loss, increased susceptibility to ischemic damage, excitotoxicity and neurodegeneration. To investigate this hypothesis *in vitro*, Ca^{2+} imaging procedures are available. Unfortunately, viable cultures of old hippocampal neurons are not reliable. Recently, it has been observed that long-term cultures of rat hippocampal neurons present many of the typical hallmarks of aging in vivo including ROS accumulation, formation of lipofuscin granules, heterochromatic foci, activation of pJNK and p53/p21 pathways, cholesterol loss, and changes the density of Ca^{2+} channels and NMDA receptors [14]. Accordingly, Ca^{2+} imaging experiments in young and old cultures of rat hippocampal neurons may provide new insights of Ca^{2+} remodeling in the aging brain. A condition that is critical for culturing hippocampal neurons in the long term is two fold. First, it is important to plate the cells at a very low density as stated above. Second, cells are cultured in supplemented Neurobasal Medium without changing the medium. This approach allows the presence of glia but avoiding their overly growth. The schematic for such culture is shown in Fig. 1.

Fluorescence imaging with synthetic Ca^{2+} probes allows the monitoring of cytosolic [Ca^{2+}] in individual neurons [19]. The use of this approach in young and aged neurons allows the monitoring of changes in the responses with age in culture. For instance Fig. 2 shows typical bright field and Ca^{2+} images of the increases in $[Ca^{2+}]_{cyt}$ induced by NMDA in young neurons and neurons from aged cultures. Notice that responses in aged neurons are much larger than in young neurons. However, this procedure is not reliable for [Ca^{2+}] measurements within subcellular organelles as, for instance, mitochondria, where [Ca^{2+}] may vary from below the µM to above the mM level. In those cases, protein based, mitochondria-targeted probes have been developed as, for instance, aequorin. This probe is particularly interesting since its affinity for Ca^{2+} can be finely tuned to monitor very low or very high [Ca^{2+}] like the ones reached inside mitochondria in resting and stimulated conditions, respectively. Specifically, it is possible to select either the wild type aequorin or a mutated aequorin without Ca^{2+} binding sites to modify the affinity. Fine tuning is achieved when different coelenterazines (wild type, h, n) are used in combination with different aequorins [17]. However, whereas the use of fluorescence for Ca^{2+} imaging is widespread, monitoring bioluminescence is not straightforward and may require photon counting cameras. Fortunately, aequorin probes, like the one used here, have been improved to co-express GFP [18] making them more stable, able to release more photonic emissions and allowing simple identification of transfected cells by the GFP associated fluorescence. Success in bioluminescence imaging in neurons depends not only on the efficiency of the photon counting camera used but also on the efficiency of the transfection of the mitochondria-targeted probe. In the case of hippocampal neurons, a simple chemical transfection may work provided that a high sensitive photon

counting camera is available. Fig. 2 shows examples of bright field, GFP fluorescence and bioluminescence images of young and aged in culture hippocampal neurons. Also shown are the increases in mitochondrial [Ca^{2+}] induced by NMDA recorded in transfected hippocampal neurons. Notice that the effect of NMDA is much larger in aged cultured neurons than in young neurons. In other cases, the efficiency of the transfection can be increased by using virus-derived vectors [16,18]. Alternatively, the development of transgenic mice harboring protein-based, subcellular Ca^{2+} probes is an emerging option for future approaches, perhaps even *in vivo*.

Disclosures

None of the authors have competing interests or conflicting interests.

Acknowledgements

This work was supported by Ministerio de Economìa y competitividad (BFU2012-37146) and Junta de Castilla y Leòn (BIO103/VA45/11, VA145U13 and BIO/VA33/13), Spain. MCR was supported by Junta de Castilla y Leòn (Spain) and the European Social Fund. We thank the late Dr. Philippe Brûlet (1947-2013) for the mitochondrial GFP Aequorin plasmid.

References

1. Sattler, R., & Tymianski, M. Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. *Molecular Neurobiology.* **24** (1-3), 107-129, (2001).
2. MacDermott, A. B., Mayer, M. L., Westbrook, G. L., Smith, S. J., & Barker, J. L. NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurones. *Nature.* **321** (6069), 519-522, (1986).
3. Choi, D. W., Maulucci-Gedde, M., & Kriegstein, A. R. Glutamate neurotoxicity in cortical cell culture. *The Journal of Neuroscience.* **7** (2), 357-368 (1987).
4. Kure, S., Tominaga, T., Yoshimoto, T., Tada, K., & Narisawa, K. Glutamate triggers internucleosomal DNA cleavage in neuronal cells. *Biochemical and Biophysical Research Communications.* **179** (1), 39-45 (1991).
5. Calvo, M., Sanz-Blasco, S., Caballero, E., Villalobos, C., & Nunez, L. Susceptibility to excitotoxicity in aged hippocampal cultures and neuroprotection by non-steroidal anti-inflammatory drugs: role of mitochondrial calcium. *Journal of Neurochemistry.* **132**(4), 403-417, (2015).
6. Liu, Y. *et al.* NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo. *The Journal of Neuroscience.* **27** (11), 2846-2857, (2007).
7. Zhou, M., & Baudry, M. Developmental changes in NMDA neurotoxicity reflect developmental changes in subunit composition of NMDA receptors. *The Journal of Neuroscience.* **26** (11), 2956-2963, (2006).
8. Brewer, L. D. *et al.* Increased vulnerability of hippocampal neurons with age in culture: temporal association with increases in NMDA receptor current, NR2A subunit expression and recruitment of L-type calcium channels. *Brain Research.* **1151**, 20-31, (2007).
9. Berridge, M. J. Calcium signalling remodelling and disease. *Biochemical Society Transactions.* **40** (2), 297-309, (2012).
10. Stout, A. K., Raphael, H. M., Kanterewicz, B. I., Klann, E., & Reynolds, I. J. Glutamate-induced neuron death requires mitochondrial calcium uptake. *Nature Neuroscience.* **1** (5), 366-373, (1998).
11. Pivovarova, N. B. *et al.* Excitotoxic calcium overload in a subpopulation of mitochondria triggers delayed death in hippocampal neurons. *The Journal of Neuroscience.* **24** (24), 5611-5622, (2004).
12. Morris, R. G., Garrud, P., Rawlins, J. N., & O'Keefe, J. Place navigation impaired in rats with hippocampal lesions. *Nature.* **297** (5868), 681-683 (1982).
13. Geinisman, Y., Detoledo-Morrell, L., Morrell, F., & Heller, R. E. Hippocampal markers of age-related memory dysfunction: behavioral, electrophysiological and morphological perspectives. *Progress in Neurobiology.* **45** (3), 223-252 (1995).
14. Sodero, A. O., Weissmann, C., Ledesma, M. D., & Dotti, C. G. Cellular stress from excitatory neurotransmission contributes to cholesterol loss in hippocampal neurons aging in vitro. *Neurobiology of Aging.* **32** (6), 1043-1053, (2011).
15. Brini, M. *et al.* Transfected aequorin in the measurement of cytosolic Ca^{2+} concentration ([Ca^{2+}]c). A critical evaluation. *The Journal of Biological Chemistry.* **270** (17), 9896-9903 (1995).
16. Villalobos, C., Alonso, M. T., & Garcìa-Sancho, J. Bioluminescence imaging of calcium oscillations inside intracellular organelles. *Methods in Molecular Biology.* **574**, 203-214, (2009).
17. Villalobos, C. *et al.* Redistribution of Ca^{2+} among cytosol and organella during stimulation of bovine chromaffin cells. *FASEB Journal.* **16**, 343-353, (2002).
18. Rogers, K. L. *et al.* Visualization of local Ca^{2+} dynamics with genetically encoded bioluminescent reporters. *The European Journal of Neuroscience.* **21** (3), 597-610, (2005).
19. Barreto-Chang, O.L., & Dolmetsch, R.E. Calcium imaging of cortical neurons using Fura-2 AM. *Journal of Visualized Experiments.* **23** pii: 1067, (2009).

