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Abstract	IntracellularCa2+ is involved in control of a large variety of cell functions including apoptosis and neuron cell death. For example, intracellular Ca2+ overload is critical in neuron cell death induced by excitotoxicity. Thus, single cell monitoring of intracellular Ca2+ concentration ([Ca2+]cyt) in neurons concurrently with apoptosis and neuron cell death is widely required. Procedures for culture and preparation of primary cultures of hippocampalratneurons and fluorescence imaging of cytosolic Ca2+concentration in Fura2/AM-loaded neurons are described. We also describe a method for apoptosis detection by immunofluorescenceimaging. Finally, a simple method for concurrent measurements of [Ca2+]cyt and apoptosis in the same neurons is described.

Keywords (separated by “-”)	Calcium imaging - Neuron death - Apoptosis - Hippocampalneurons - Fura2/AM

Chapter 6

[AU1] Calcium Imaging in Neuron Cell Death

[AU2] María Calvo, Carlos Villalobos, and Lucía Núñez

Abstract

Intracellular Ca2+ is involved in control of a large variety of cell functions including apoptosis and neuron cell death. For example, intracellular Ca2+ overload is critical in neuron cell death induced by excitotoxicity. Thus, single cell monitoring of intracellular Ca2+ concentration ([Ca2+]cyt) in neurons concurrently with apoptosis and neuron cell death is widely required.

Procedures for culture and preparation of primary cultures of hippocampal rat neurons and fluorescence imaging of cytosolic Ca2+ concentration in Fura2/AM-loaded neurons are described. We also describe a method for apoptosis detection by immunofluorescence imaging. Finally, a simple method for concurrent measurements of [Ca2+]cyt and apoptosis in the same neurons is described.

Key words Calcium imaging, Neuron death, Apoptosis, Hippocampal neurons, Fura2/AM

1 Introduction

Intracellular free calcium concentration ([Ca2+]cyt) plays a pivotal role in control of a large variety of cell and physiological functions in most cells and tissues from the very short term (neurotransmitter release, muscle contraction) to the long-term scale (gene expression, cell proliferation). In general, an increase in [Ca2+]cyt triggers cell activation. However, if the rise in [Ca2+]cyt is large and/or sustained enough, it promotes rather cell death in many cell types including neurons [1]. Examples include overly activation of ionotropic glutamatergic receptors during excitotoxicity, particularly N-methyl D-aspartate (NMDA) [2, 3] as well as Ca2+ overload induced by amyloid β oligomers in Alzheimer´s disease [4]. These events may be followed by a delayed [Ca2+]cyt increase long after stimuli removal and lead to calpain activation, turning on a proteolytic cascade culminating in neuron cell death [5]. This process is usually limited by endogenous buffers including proteins like calbindin and organelles like mitochondria. However, if mitochondria take up too much Ca2+, they favor the delayed rise in [Ca2+]cyt [6] as well as the opening of the so-called mitochondrial

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permeability transition pore, a not well understood phenomenon that precedes release of cytochrome c and other pro-apoptotic factors culminating in apoptosis [7]. Therefore, changes in neuronal [Ca2+]cyt are critical in neuron apoptosis and cell death. In the present chapter, a detailed description of methods for primary culture of rat hippocampal neurons is provided together with means for imaging [Ca2+]cyt rises in neurons and apoptosis formation in the same cultures. Finally, a concurrent procedure is presented for monitoring both Ca2+ levels and apoptosis in the same single neurons.

2 Materials

2.1 Primary Culture of Rat Hippocampal Neurons

1. Neonatal Wistar rats. *See* **Note 1**.
2. HEPES-buffered saline (HBS): 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM sodium-HEPES, pH 7.4. Use double distilled water for preparation.
3. 4 % bovine serum albumin (BSA) in Hanks balanced salt solution (HBSS) without Ca2+ and Mg2+ (Gibco®, Life Technologies™, Gaithersburg, MD): dissolve 4 g of BSA in 100 mL HBSS.
4. Hank's medium + 0.6 % BSA: mix 85 mL HBSS without Ca2+ and Mg2+ (Gibco®, Life Technologies™) with 15 mL 4 % BSA in HBSS.
5. Papain (Worthington, Lakewood, NJ): Prepare cell dissociation solution diluting the enzyme at 20 U/mL in Hank's + 0.6 % BSA.
6. Deoxyribonuclease I (DNase I) from bovine pancreas (Sigma Chemicals, St. Louis, MO): Dissolve DNase in Hank´s medium + 0.6 % BSA to a final concentration of 1 mg/mL.
7. Neurobasal® Culture Medium (Gibco®) with 10 % fetal bovine serum (FBS, Lonza, Basel, Switzerland), 2 % B27 (Gibco®), 1 μg/mL gentamicin (50 mg/mL, Gibco®) and 2 mM L-glutamine (Gibco®).
8. 12 mm glass coverslips (Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany).
9. Poly-D-lysine (Becton Dickinson, Franklin Lakes, NJ).
10. 4-Well multidish plaques for 12 mm glass coverslips (Nunc, Rochester, NY).
11. 5 and 10 mL sterile pipettes (Fisher Scientific, Loughborough, UK).
12. HeraCell 150 Incubator (Thermo Scientific, Waltham, MA).
13. Centrifuge.
14. Neubauer counting chamber.

2.2 Calcium Imaging

1. HEPES-buffered saline (HBS): 145 mM NaCl, 5 mM KCl, 1 Mm $MgCl_2$, 1 Mm $CaCl_2$, 10 Mm glucose, 10 mM sodium-HEPES, pH 7.4.
2. Fura2/AM (Invitrogen™, Life Technologies™, Gaithersburg, MD): 2 mM stock solution in DMSO. Store at – 20 °C.
3. Inverted fluorescence microscope *or* confocal microscope. *See* **Note 2**.
4. Cell perfusion system for living cells. *See* **Note 3**.
5. Light-proof box covering the imaging setup (about 100 × 100 × 100 cm): Also available from commercial sources, e.g., Hamamatsu Photonics.

2.3 Excitotoxicity and Apoptosis Assays

1. Mg2+-free, HEPES buffered saline: 146 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 10 mM glucose, 10 mM sodium-HEPES, pH 7.42.
2. *N*-methyl-D-aspartic acid (NMDA), e.g., Sigma Chemicals.
3. Glycine.
4. FITC-conjugated Annexin V (Becton Dickinson).
5. Annexin V binding buffer: 140 mM NaCl, 2.5, $CaCl_2$, 10 mM sodium-HEPES, pH 7.4.
6. Fluorescence microscope, e.g., Nikon Eclipse TS100 microscope (objective 40×) coupled with fluorescence filters (Nikon, Tokyo, Japan).

3 Methods

3.1 Primary Rat Hippocampal Neuron Cell Culture

1. Sterilize 12 mm glass coverslips in ethanol for 24 h, and allow drying under sterile conditions.
2. Cover one side of the coverslip overnight with approximately 200 µL of 1 mg/mL poly-D-lysine solution.
3. On the next day wash coverslips with double distilled sterile water every 15 min for 90 min under sterile conditions.
4. Place coverslips in a 4-well multidish plaque filled with 500 µL of Neurobasal® Culture Medium, and maintain in a humidified 37 °C incubator with 5 % CO_2 until use. Treated coverslips can be used for up to 1 week.
5. Obtain hippocampal neurons from two newborn Wistar rat pups [4, 9]. Kill newborn rat pups by decapitation, and quickly wash the head in sterile HBS medium. Then, open the skull to extract the brain, and wash it quickly with sterile HBS medium before dissecting the hippocampus.
6. Make a diagonal cut with a scalpel in each hemisphere, and transfer the top side of each hemisphere to a Petri dish containing

sterile HBS medium. With the help of a magnifying glass, carefully remove the meninges and separate the hippocampi from the cortex.

7. Wash hippocampal tissue with sterile Hank's medium without Ca2+ or Mg2+ + 0.6 % BSA and then cut it into small pieces of about 2 × 2 mm.
8. Transfer small hippocampal pieces to a 10 mL centrifuge tube containing 10 mL of pre-filtered papain solution. Close the tube and put inside the 37 °C incubator. After 15 min incubation, add 500 µL of DNase I solution (50 µg/mL final concentration) and further incubate at 37 °C for another 15 min with occasional, gentle shaking.
9. Wash tissue fragments three times with fresh Neurobasal® Culture Medium. To do so, allow pieces to go to the bottom of the tube (by gravity) and remove medium. Then add 5 mL sterile Neurobasal® Culture Medium and gently shake the suspension. Repeat this procedure is two more times.
10. Disperse tissue fragments into a cell suspension using a 5 mL plastic pipette. Specifically, after the last wash, add 3 mL Neurobasal® Culture Medium and disperse pieces by passing them 10–12 times through a 5 mL plastic pipette. Allow small pieces to set by gravity and collect the 3 mL medium in another 10 mL centrifuge tube. Repeat this procedure is three times.
11. Centrifuge the cell suspension gently (160 × *g*, 5 min). Remove the supernatant and suspend the cell pellet is in 1,000 µL Neurobasal® Culture Medium.
12. Estimate cell density using a Neubauer counting chamber. Add 30 × 103 cells (around 50–70 µL cell suspension) to each well previously filled with poly-D-lysine coated coverslips in 500 µL Neurobasal® Culture Medium.
13. Maintain hippocampal cells in culture for 14–16 days in vitro (DIV, *see* **Note 4**) in a humidified incubator at 37 °C and 5 % CO_2 (*see* **Note 5**) before experiments. Figure 1 shows bright field pictures of rat hippocampal neurons cultured for 2, 8 and >14 DIV as well as the typing of glia and neurons by specific immunofluorescence (*see* **Note 6**) for details on identification of glia and neurons by twofold immunofluorescence microscopy.

3.2 Fluorescence Imaging of Cytosolic Ca2+

1. Wash coverslips containing cultured hippocampal cells with HBS medium twice and incubate in the same medium containing 4 µM Fura2/AM for 60 min at room temperature and in the dark.
2. Place coverslips on a thermostatic platform for open 12 mm glass coverslips (total volume about 500 µL) on the stage of the inverted microscope.

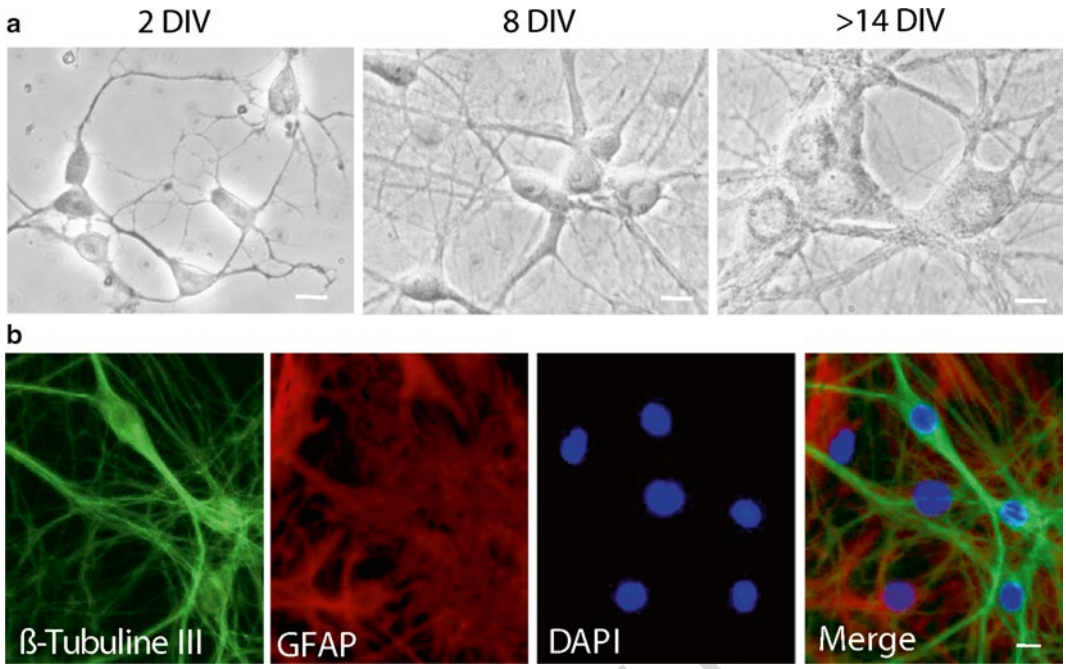

Fig. 1 Primary rat hippocampal neurons in culture. (**a**) Representative microphotographs of neurons as they grow and develop in culture. It can be appreciated that processes are not completely developed until around 8 days in vitro (DIV). Bars represent 10 μm. (**b**) Images of a double immunocytochemistry on a mixed primary culture of hippocampal cells, where neurons labeled with β-tubulin III (*green*) and glia labeled with GFAP (*red*) are appreciated. Nuclei are stained with DAPI. *Top right* image shows the merge of the above fluorescent images. Bar represents 10 μm

3. Continuously perfuse cells in the platform containing coverslip with heated (37 °C) HBS medium at a rate of about 5–10 mL/min. *See* **Notes 3** and 7.
4. Found focal plane before searching for a representative microscopic field that should contain ideally at least 5–10 neurons and glial cells for imaging (Fig. 1).
5. Once the microscopic field is selected, insert excitation filters for Fura2/AM imaging (340 and 380 nm) and test fluorescence emission at 520 nm using standard conditions for Ca2+ imaging: camera exposure time of about 100 ms for each wavelength, camera gain (about 50 %), offset (about 10 %) and binning (2 × 2). These conditions allow having good quality signal to noise ratio. Change settings as required according to the quality of the signal. S*ee* **Note 8**.
6. Epi-illuminate cells alternately at 340 and 380 nm using band pass filters located on the excitation filter wheel. Light emitted above 520 nm at both excitation lights is filtered by the Fura-2 dichroic mirror, collected every 5–10 s with a 40×, 1.4 NA, oil objective and recorded using the CCD camera.

7. Capture a background image at both excitation wavelengths with the shutter closed. During recordings, perfuse cells either with heated (37 °C) control HBS or HBS containing test substances at a flow of 5 mL/min. This flow ensures that the medium bathing the cells is exchanged about ten times in less than 1 min. *See* **Note 7**.
8. At the end of the recording period, store in the computer for further analysis the complete sequences of images emitted at 520 nm after 340 and 380 nm excitation light.
9. Use the Aquacosmos software, to subtract background images and calculate the pixel by pixel ratio in the resulting images to obtain a sequence of ratio images. Code ratio images in pseudocolor to better appreciate changes in Ca2+ concentrations. Perfusion of hippocampal neurons with agonists such as 100 μM NMDA or high K+ (145 mM) induces rather dramatic increases in these ratios (Fig. 2).

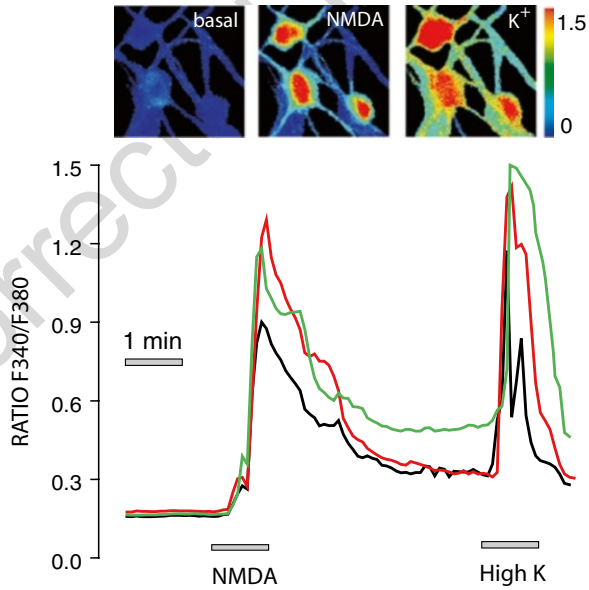

Fig. 2 NMDA and high-K+ medium increased cytosolic [Ca2+] in neurons. Hippocampal cells were cultured, loaded with Fura2/AM and subjected to Fura2 imaging. Pictures show pseudocolor images (Ratio F340/F380) of hippocampal neurons before (basal, *left*) and after stimulation with either 100 μM NMDA (NMDA, middle) or high K+ (K+, *right*). Warmer colors reflect increased cytosolic Ca2+ concentration (pseudocolor scale is shown at *right*). Recordings show cytosolic Ca2+ concentrations (Ratio F340/F380) averaged for a region of interest (ROI) corresponding to each individual neuron and taken every 5 s in three individual cells. Perfusion of HBS medium containing 100 μM NMDA or 145 mM K+ solutions increased cytosolic Ca2+ concentrations in all three neurons

10. For quantitative analysis of individual cells, draw regions of interest (ROIs) on individual cells and average all ratio values corresponding to all pixels within each ROI for each image resulting in a recording of ratio values for individual ROIs (cells).
11. Recorded ratio values can be converted into Ca2+ concentration values using the algorithm developed by Grynkiewicz et al. [10]. *See* **Note 9**.
12. For analysis of differences, express changes in fluorescence ratio as area under curve (AUC). Perform calculation of AUC using Origin Lab 7.0. Curves are defined as the period between which fluorescence ratio significantly exceeded and returned to the basal level following a stimulus.

3.3 Excitotoxicity and Apoptosis Assessment

1. Wash primary rat hippocampal neurons in culture with HBS and then treat them for 1 h with or without NMDA (100 μM) in Mg2+-free, HBS pH 7.42 supplemented with 10 μM glycine. During this time keep cells are kept at 37 °C.
2. After NMDA treatment, wash coverslips containing hippocampal neurons in HBS and then return them to the original Neurobasal® Culture Medium and culture for 24 h in the incubator at 37 °C and 5 % CO_2.
3. Wash cells once with PBS.
4. Test for apoptosis by incubating cells for 10 min with Annexin V diluted 1:20 in Annexin V binding buffer.
5. Assess staining (apoptosis) by fluorescence microscopy using a 40× objective.
6. For quantitative analysis, evaluate the percentage of apoptotic cells by counting the number of Annexin V-positive cells (green fluorescent channel), then dividing by the total number of cells in the field and multiplying by 100. Choose at least four microscopic fields randomly for a total of at least 60 neurons for each coverslip. Score two coverslips per condition in each experiment (Fig. 3).

3.4 Combination of Cytosolic Ca2+ Imaging and Annexin V Staining

This procedure is intended to combine the two methods described above (Subheadings 3.2 and 3.3) concurrently on the very same cells. The method, although time consuming, may allow correlating Ca2+ changes with susceptibility to apoptosis in the same cells.

1. Incubate coverslips containing cultured hippocampal cells in HBS medium containing 4 μM Fura2/AM for 60 min and then subject to calcium imaging as described before (*see* Subheading 3.2).
2. Perfuse hippocampal neurons with apoptosis-inducing agonists such as NMDA and record rises in [Ca2+]cyt in real time.

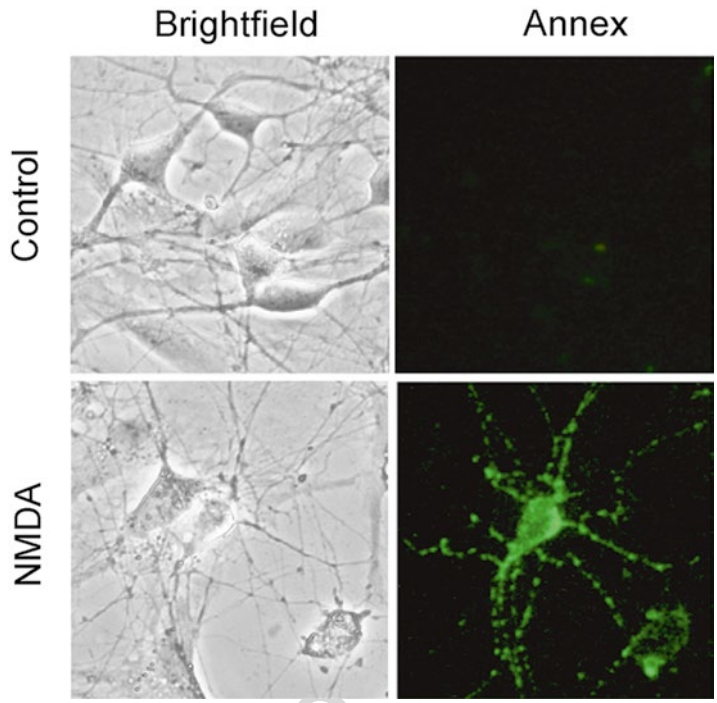

Fig. 3 NMDA induces apoptosis in neurons. Representative microphotographs of cultured hippocampal neurons in control conditions or treated with 100 μM NMDA. Hippocampal neuron cells were treated for 1 h in absence (control) or presence of NMDA (100 μM), and apoptosis was assessed 24 h later by staining with Annexin V. Microphotographs show phase contrast images (*left*) and the green fluorescence staining (Annexin V, *right*) of apoptotic cell bodies. Bars represent 10 μm

Duration of the stimulation depends on the stimulus. For NMDA a 60–70 min period is typically used. During that period, cut off for 10 min excitation light to limit cell damage by UV light (*see* straight line gaps in Fig. 4).

3. At the end of the recording period, store the sequences of images emitted at 520 nm after 340 and 380 nm excitation light in the computer. Analyze them later using the Aquacosmos software.
4. Wash cells with prewarmed HBS at 37 °C during 10 min to remove the stimulus. Then keep in the same medium at room temperature for several hours before assessing apoptosis in the same microscopic field. *See* **Note 10**.
5. With extreme care to avoid moving the microscopic field, incubate cells for 10 min in the platform with Annexin V diluted 1:20 in Annexing V binding buffer 1× (*see* Subheading 3.3). Carry out addition of Annexing V by emptying carefully the chamber and by adding drops of Annexin V solution to the coverslip.

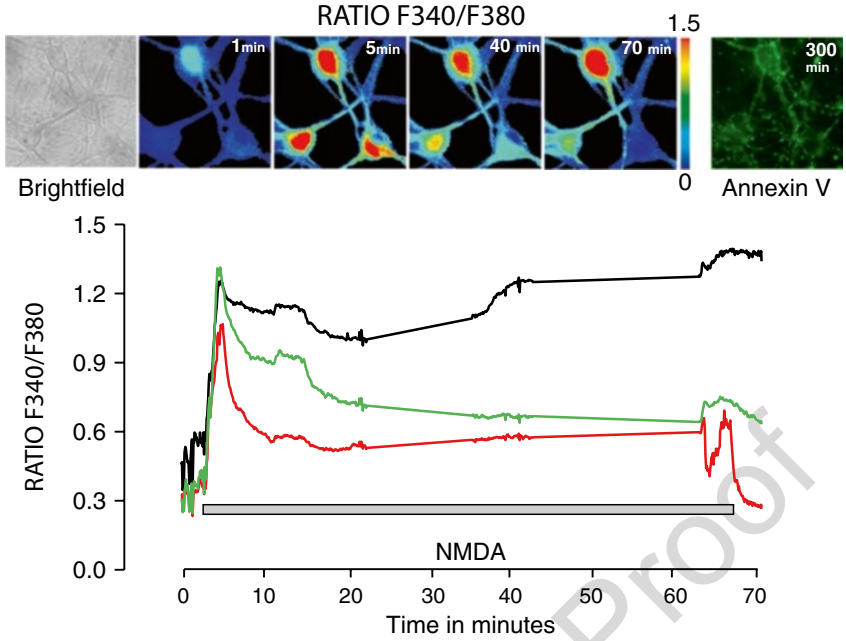

Fig. 4 Concurrent imaging of NMDA-induced cytosolic Ca2+ increases and apoptosis in the same neurons. Pictures show a bright field (*left*), pseudocolor images (Ratio F340/F380) before (basal) and at different times after stimulation with 100 μM NMDA. Annexin V staining of the same cells after several hours of treatment is shown at right. Picture shows the time from the beginning of the stimulus presentation to apoptosis assessment, in this particular case 300 min (5 h). Recordings show cytosolic Ca2+ concentrations (Ratio F340/F380). Perfusing cells with a solution containing 100 μM NMDA for 60 min increased [Ca2+]cyt in all three neurons and induced apoptosis in some of them. During recording, excitation light is cut off for 10 min periods (*straight lines* in records) to limit cell damage by UV light

6. Wash Annexin V with HBS solution and assess staining by fluorescence in the same microscopic field using a 40× objective (Fig. 4).
7. If required, correlate analysis of Ca2+ values with apoptosis induction at a given time in the same ROIs (cells).

4 Notes

1. Animals must be obtained from an approved facility and protocols must be approved by ad hoc institutional body. In our case rats were obtained from the Valladolid University animal facility and 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.

2. The set-up in use is made of a Zeiss Axiovert S100 TV inverted microscope (Carl Zeiss Inc., Gottingen, Germany) equipped with a Zeiss Fluar 40×, 1.3 NA oil objective, a Xenon XBO75 fluorescence excitation lamp or a XCITE illumination system (EXFO, Ontario, Canada), an excitation filter wheel (Sutter Instrument Company, Novato, CA) with band pass filters for Fura2 excitation (340 and 380 nm) and a Fura2 dichroic mirror. Attached in the lateral port of the microscope is a Hamamatsu Orca ER Digital Camera (Hamamatsu Photonics, Hamamatsu, Japan). Camera capturing and filter wheels are handled by Aquacosmos software (Hamamatsu Photonics). A schematic of the imaging setup has been described in detail in a recent chapter [8].
3. The cell perfusion system is mounted in a PH-3 thermostatic platform for open 12 mm glass coverslips using an 8-lines gravity-driven perfusion system equipped with pinch valves (VC-8 valve controller) and solutions heated using a SH-27B inline heating system. All the above components are from Warner Instruments, Hamden, CT.
4. Hippocampal neurons require several days in vitro for establishment of neural connections and proper responsiveness to glutamate receptor agonists. Accordingly, to test for excitotoxicity it is required to culture hippocampal cells for at least 2 weeks (>14 DIV). Neuron cells cultured for less than 2 weeks may show resistance to apoptosis [11].
5. An incubator set to hold a 5 % of CO_2 is to be used when Neurobasal® Culture Medium is employed because it contains 24 mM $NaHCO_3$. In case that other culture mediums containing 44 mM (instead 24 mM) $NaHCO_3$ are used (as DMEM for instance), the incubator should be set to 10 % CO_2 to keep pH in physiological conditions (pH 7.4).
6. In order to identify glia and neurons in the culture, twofold immunofluorescence can be used. To accomplish this treat cells in the glass coverslip with 4 % paraformaldehyde (PFA) prepared in a phosphate buffer solution (PBS) for 20 min. Wash the coverslips three times with PBS. Treat them with 0.1 % Triton X-100 solution for 10 min and then wash again coverslips three times with PBS. Incubate next the cells in PBS containing 20 % goat serum for 20 min to suppress non-specific binding of antibody. Then, incubate coverslips with primary antibodies prepared in PBS containing 10 % goat serum at 1:300 dilution for 1 h. To identify neurons and glia, mouse anti-β-tubulin III and rabbit anti-glial fibrillary protein (GFAP) can be used. After 1 h incubation, wash coverslips three times with PBS before incubating cells with anti-mouse IgG FITC labeled secondary antibody (dark room), which

emits green fluorescence, and anti-rabbit IgG Alexa Fluor 594 labeled secondary antibody, which emits red fluorescence. These antibodies had also been prepared in PBS containing 10 % goat serum at a 1:300 dilution. After 45 min incubation, wash coverslips three times with PBS and incubate fixed cells for 5 min with DAPI, prepared 1:5,000 in PBS containing 10 % goat serum. Finally, place coverslips on a slide with mounting medium (50 % glycerol in PBS) and capture images in a fluorescence microscope. It is possible to carry out this procedure in the same coverslip used for Ca^{2+} imaging. In that case, do not remove coverslip from the recording chamber and add and remove solutions carefully in order to avoid losing the microscopic field. In this case, it is possible to use the same microscope for calcium imaging and immunofluorescence in exactly the same single cells.

7. Perfusion is helpful during imaging not only to quickly and easily add and remove test solutions to and from the cell chamber, but also to keep physiological conditions (37 °C or even CO_2 if required) using the in-line heating system. This is particularly true for long-term experiments where water evaporation concentrates solutes modifying physiological conditions. In the case one needs to test very expensive compounds such as, for instance, Aβ species, perfusion may be cost limiting. In those cases were perfusion is not available or convenient, solutions can be provided by carefully adding a drop of 2× test-containing solution, to a half-filled chamber and making a quick mix of solutions with the tip of the pipette. In this case, it is best to keep the heating system off and make the experiment at room temperature to avoid changes in the saline composition due to evaporation.
8. Fluorescence settings have to be adjusted in each imaging setup depending on multiple factors including excitation lamp intensity in the UV range (340 and 380 nm), quality of the lens, dichroic mirror, and quality of the camera. Usually Fura2/AM-loaded cells do not emit much light when excited at 340 nm. In those cases it may be possible to use different setting for each wavelength. In general, glass coverslips are required to have good light transmission in the UV. Avoid plastics that absorb UV light. If not enough signal is achieved, it is possible to increase gain, decrease offset, increase the binning to 4×4 pixels and also the Fura2/AM loading time.
9. Binding of Ca2+ to Fura2 changes Fura2 fluorescence according to the low of mass action [10]:

$$\left[Ca^{2+}\right] = K_d \left(F_{max} - F\right) / \left(F - F_{min}\right);$$

where K_d, is the dissociation constant of the Fura2-Ca2+ complex (224 nM at 37 °C); F is the fluorescence emission for each [Ca2+]; F_{max}, is the fluorescence emission when Fura2 is saturated with Ca2+ and Fmin is fluorescence emission when Fura2 is free of Ca2+. If we apply the above algorithm to both wavelengths and do the ratio, then we obtain the following algorithm:

$$\left[Ca^{2+}\right] = K_d \beta \left(R_{max} - R\right) / \left(R - R_{min}\right);$$

where R is the ratio of fluorescence recordings obtained after exciting at 340 and 380 nm for a given [Ca2+]; R_{max}, is the same ratio when Fura2 is saturated with Ca2+ and R_{min} is the same ratio when Fura2 is free of Ca2+. Finally, β is the ratio of F_{max} / F_{min} at 380 nm. This algorithm allows estimation of [Ca2+] knowing the R values at any point in time. R_{max}, R_{min} and β values can be determined experimentally using Fura2/AM solutions in the presence of saturating concentrations of Ca2+ (HBS containing 1 mM Ca2+) and in the absence of Ca2+ free medium (HBS without added Ca2+ and containing EGTA 5 mM).

10. Usually, this kind of cell death assays are performed several hours after treatment because the stimulus induce a delayed cell death [6], and it involves events during or shortly after the acute phase of stimulation that would detonate the death sequence. During this time cells are kept in HEPES-containing buffer at room temperature to limit water evaporation and changes in saline. Alternatively, the whole experiment could be carried out in an incubator attached on the microscope's platform and carried out in $NaHCO_3$ buffered medium and at 37 °C. There are commercially available incubators for inverted microscopes from Carl Zeiss Inc., Gottingen, Germany.

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