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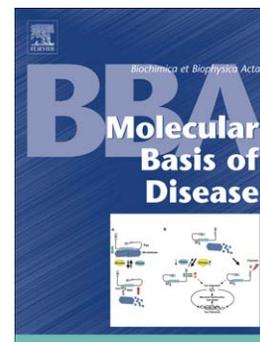
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Lipid raft disruption protects mature neurons against amyloid oligomer toxicity

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Summary

A specific neuronal vulnerability to amyloid protein toxicity may account for brain susceptibility to protein misfolding diseases. To investigate this issue, we compared the effects induced by oligomers from salmon calcitonin (sCTOs), a neurotoxic amyloid protein, on cells of different histogenesis: mature and immature primary hippocampal neurons, primary astrocytes, MG63 osteoblasts and NIH-3T3 fibroblasts. In mature neurons, sCTOs increased apoptosis and induced neuritic and synaptic damages similar to those caused by amyloid β oligomers. Immature neurons and the other cell types showed no cytotoxicity. CTos caused cytosolic Ca^{2+} rise in mature, but not in immature neurons and the other cell types. Comparison of plasma membrane lipid composition showed that mature neurons had the highest content in lipid rafts, suggesting a key role for them in neuronal vulnerability to sCTOs. Consistently, depletion in gangliosides protected against sCTO toxicity. We hypothesize that the high content in lipid rafts makes mature neurons especially vulnerable to amyloid proteins, as compared to other cell types; this may help explain why the brain is a target organ for amyloid-related diseases.

INTRODUCTION

A number of degenerative disorders are pathogenetically linked to a process of misfolding and aggregation of specific proteins, the large family of the so-called amyloid proteins [1-3]. The process of aggregation that leads to the characteristic cytotoxic properties generally proceeds through the formation of dimers, oligomers and protofibrils, before finally assuming an insoluble fibrillar conformation, characterized by β -structures. It is now generally agreed that the cytotoxic species are the soluble, oligomeric aggregates, while the insoluble fibrils seem to be relatively non-harmful. Small oligomeric assemblies of Amyloid β (A β) for example have been specifically shown to impair long-term potentiation and dendritic spine structure in the hippocampus and to disrupt cognition in animals (For a review see Nimmrich and Ebert, 2009 [4]). Several findings suggest that a common pathologic pathway exists in the case of amyloid associated neurodegenerative diseases, which is independent from the peptide primary sequence [5,6]. According to this hypothesis, neurotoxicity of extracellular amyloid aggregates is due to the formation of Ca^{2+} -permeable channels by incorporation of amyloid protein oligomers in the plasma membrane, resulting in an imbalance in Ca^{2+} inflow and consequent cell death [5,7-9].

Calcitonin is a polypeptidic hormone secreted by the thyroid gland and, due to its typical aggregation behaviour, belongs to the amyloid family [10,11]. Even if it is not involved in any neurodegenerative diseases, aggregated CT is toxic to neurons in vitro [12-15], like other amyloid proteins.

Salmon Calcitonin (sCT), found to be neurotoxic as CT from other species [12,13, 16], is characterized by a slower aggregation rate [16] and this peculiarity is at the basis of its pharmacological use. This observation that may provoke concern, since the possibility that

protein aggregates may reach the brain from the blood circulation and trigger a neurotoxic reaction should be taken into consideration, especially considering CT is often delivered as a nasal spray [17]. We have recently shown that sCT Oligomers (sCTOs) forms Ca^{2+} -permeable pores in liposomes [18], highly reminiscent of the ion channels formed by other amyloid proteins, such as $\text{A}\beta$ and α synuclein [6,9,19,20]. We have also shown that sCTO insertion in model membranes occurs only in the presence of GM1 ganglioside and cholesterol, which are typical components of lipid rafts [21]. A large body of evidence sustains a role for lipid rafts in amyloid-induced neurotoxicity. Lipid rafts have been shown to be involved in $\text{A}\beta$ formation [22,23]. Of particular interest is their involvement in the mechanisms by which amyloid oligomers interact with neuronal plasma membrane and trigger a neurotoxic response. It has been reported that the neurotoxicity of some amyloid proteins, such as $\text{A}\beta$ peptides and calcitonin, is strongly reduced by the depletion of ganglioside and/or cholesterol [14,15,24]. In addition, it has been shown that $\text{A}\beta$ binds to sialic acid-containing gangliosides [25,26]. The aims of the present study were i) to compare the cytotoxic response to sCTOs of cultured hippocampal neurons to that observed in different cell types with the aim of determining if a diverse sensitivity exists; ii) to investigate if sCTO-induced neurotoxic response is related to an imbalance of intracellular Ca^{2+} ; iii) to characterize the role played by lipid rafts in sCTO toxicity.

MATERIALS AND METHODS

Preparation of sCTO and A β solutions

Lyophilized sCT (European Pharmacopoeia, EDQM, France) was stored at -18°C before use. sCTO 1 mM solution was prepared by dissolving the protein in 5 mM phosphate buffer, pH 7.4 and incubated at room temperature for 3 h. No stock solutions of protein were employed. After 3 h, sCT solutions, containing sCTOs, were diluted to a final concentration of 80 μM for CD, TEM, neurotoxicity and Ca^{2+} influx experiments and 25, 60, 80 or 120 μM for growth curves. To analyze sCT effects in its monomeric form, sCT was diluted in deionized water (1mM) immediately before use. A β oligomers were prepared as described by Stine et al. [27]. Briefly, lyophilized 1-42 A β peptide, (Bachem, Switzerland), was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma, USA) and dried. Before use, an aliquot was dissolved in dimethyl sulfoxide (5mM), brought to 1mM in Minimum Essential Medium (MEM, Invitrogen, Italy), incubated at 4°C for 24 h. The final dilution was 20 μM .

Transmission Electron Microscopy

Negative staining was obtained using a phosphotungstic acid (PTA) 2% w/v solution, buffered at pH 7.3 with NaOH. To avoid salt precipitation from PTA and/or NaOH, which can be misinterpreted as actual structures, the staining solution was filtered before each preparation through polycarbonate 0.2 μm pore filters. A droplet of protein solution was deposited onto 400-mesh copper grids for electron microscopy and covered with a thin amorphous carbon film (about 20 nm). When the grid was dried, a droplet of PTA was deposited and let dry. The samples were studied at a Zeiss 902 Transmission Electron Microscope, operating at 80 kV,

equipped with an Electron Energy Loss filter. In order to enhance the contrast, the microscope was used in the Electron Spectroscopy Imaging mode filtering at $\Delta E=0$ eV. The image acquisition was performed by a digital HSC2 CCD camera, 1k for 1k pixels, (Proscan gmbh, Germany), thermostatted by a Peltier cooler model WKL 230 (Lauda gmbh, Germany). Image analysis and quantification was performed by the digital image analyzer analySIS 3.0 (Sis gmbh, Germany). This software allows to enhance contrast and sharpness of the acquired images and to perform morphological quantification and statistics. The dimensional measurements were performed after a careful magnification calibration of the whole imaging system based on reference standards.

Circular Dichroism (CD) measurements

CD measurements were performed on a Jasco J-715 spectropolarimeter (Jasco Corporation, Japan) in the far-UV region (260-190 nm). All spectra, mean of 4 different scans, were blank subtracted. Quartz cells of 0.1 cm path-length were employed. The spectral resolution was 0.1 nm and the speed scan 50 nm/min. All CD spectra were reported as $De = [\Theta] / 3300$, where $[\Theta]$ is equal to $(\theta \times 100)/(l \times C)$ (θ is the measured ellipticity, C is the molar amino acidic concentration ($=8 \times 10^{-5} \times 32M$) and l is the pathlength in cm). CD spectra were analyzed quantitatively by a fitting procedure performed using the CDSSTR software available at the website of the Birkbeck College of the University of London.

Cell cultures

Primary hippocampal neurons were prepared from embryonic-day-18 rat brain, according to the method of Brewer et al. [28], slightly modified [29]. All experimental procedures were in line with the “Ethical principles and guidelines for scientific experiments on animals” of the

Swiss Academy of Medical Sciences and national laws. After dissection, the hippocampi were treated with 2.5% trypsin and dissociated. The cells were plated in 24-well plates, containing poly-L-lysine-treated glass coverslips, in MEM, containing 10% heat-inactivated Fetal Calf Serum (FCS). After 2 h, the cell culture medium was substituted with Neurobasal medium with B27 supplement (NBM/B27, Invitrogen, Italy), 700 μ l for well. At Day-*In-Vitro* (DIV) 1, 5 μ M arabinosylcytosine was added. Neuronal cell cultures contained less than 1% of astrocytes, as shown by glial fibrillary acidic protein staining (data not shown). Hippocampal neurons were used at DIV6 (immature neurons) and 14 (mature neurons). Primary astrocytic cultures were obtained from embryonic-day-18 rat brain cortex and cultivated in MEM/FCS. Cultures were trypsinized and replated twice before use. MG-63 cells (human osteosarcoma cell line), purchased from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia (Brescia, Italy), were grown in monolayer in tissue culture flasks containing RPMI (Invitrogen, Italy) supplemented with 10% FCS. NIH-3T3 (mouse embryo fibroblasts), purchased from the American Type Culture Collection (ATCC), were grown in monolayer in tissue culture flasks containing DMEM (Invitrogen, Italy) supplemented with 10% FCS.

Treatments

For apoptosis detection and immunolabeling, 300 μ l of cell culture medium from each coverslip-containing wells were removed and kept in the incubator (conditioned medium). The cells were given sCTOs or A β for 20 min. The cell culture medium was then removed and substituted with the conditioned medium and the cells fixed after 24 h. For evaluation of intracellular Ca²⁺, sCTOs were given directly to the wells on the microscope stage, after 10 min recording. For sialic acid depletion, mature hippocampal neurons were treated with a

neuraminidase (NAA) cocktail, composed of 11.7 milliunits of *Vibrio cholerae* NAA and 3.3 milliunits of *Arthrobacter ureafaciens* NAA (Sigma), in medium for 1h at 37 °C prior to sCTOs. For cholesterol depletion, cells were treated with 2 mM MCDX (Sigma) for 45 min at 37 °C prior to sCTOs. For defining the role of the NMDA receptor in sCTOs-induced changes in intracellular Ca^{2+} , in some experiments hippocampal neurons were pre-treated with 2 μM thapsigargin (10 min prior to sCTOs) or 10 μM MK801 (5 min prior to sCTOs) or one of the following antibodies, 45 min prior to sCTOs: polyclonal anti-NR1, (1:100, Upstate Biotechnology), polyclonal anti GM1 (1:100, Chemicon), polyclonal anti-Bovine Serum Albumin (BSA), 100. $\mu\text{g}/\text{ml}$, kind gift of Paola Margutti.

Immunocytochemistry

Cell cultures were fixed in 4% paraformaldehyde in PBS, 0.12M in sucrose, and permeabilized with Triton X-100 (0.2%, Sigma). Hippocampal neurons were immunostained for Microtubule-Associated Protein 2 (MAP2), polyclonal antibody diluted 1:200 (Chemicon) and synaptophysin, monoclonal antibody diluted 1:200 (Millipore). Secondary antibodies were FITC-conjugated rabbit anti-mouse and TRITC-conjugated goat anti-rabbit IgGs (Sigma). Nuclei were counterstained with Hoechst 33258. Samples were observed at an Eclipse 80i Nikon Fluorescence Microscope, equipped with a VideoConfocal (ViCo) system.

Optical fluorimetric recordings

Optical fluorimetric recordings with Fura-2 AM were used to evaluate the intracellular Ca^{2+} concentration. Fura-2 AM stock solutions were obtained by adding 50 μg of Fura-2 AM to 50 μl of 75% DMSO plus 25% pluronic acid. Cells were bathed for 60 min at room temperature with 5 μl of stock solution diluted in 1 ml of extracellular solution (in mM: 125 NaCl, 1 KCl, 5

CaCl₂, 1 MgCl₂, 8 glucose, and 20 HEPES, pH 7.35) for a final Fura-2 AM concentration of 5 μ M. This solution was then removed and replaced with extracellular solution, and the dishes were quickly placed on the microscope stage. To measure fluorescence changes, a Hamamatsu (Shizouka, Japan) Argus 50 computerized analysis system was used, recording every 6 sec the ratio between the values of light intensity at 340 and 380 nm stimulation.

Apoptosis detection

To detect apoptosis, cells were fixed as described, stained with Hoechst 33258 and observed at the Nikon fluorescence microscope. Non-apoptotic cells exhibit a diffuse nuclear fluorescence while apoptotic nuclei appear fragmented or highly condensed. In some experiments, neurons were treated with the TUNEL technique, using the DeadEnd kit (Promega, Madison, WI) and observed at a Nikon Optiphot microscope. Differential counts of normal and apoptotic cells were performed on randomly chosen microscopic fields for a total of at least 200 cells for each coverslip. Two coverslips were scored for each condition. The values obtained for each coverslip were averaged to produce a single mean value for each experiment.

Growth curves

In order to determine cell proliferation, MG-63 and NIH-3T3 cells were seeded at 2×10^4 cells/ml of cell culture medium and incubated at 37°C in a 5% CO₂ atmosphere. After 24 h of incubation, cells were treated with 25, 60, 80 or 120 μ M sCTO for 20 min and then left to grow in the medium in absence of sCTO for 24, 48 and 72 h. Untreated MG-63 cells were considered as control. After each time of incubation, cells were detached by first adding 10 mM EDTA (pH 7.4) and, once this solution was removed, by adding 0.25% trypsin and counted by using a Neubauer chamber. Each point was conducted in triplicate.

Preparation of low-density Triton-insoluble (LDTI) plasma membrane domains

The different cell types were fractionated using a detergent (1% Triton X-100, Sigma)-containing method [30]. The cells were washed in ice cold PBS twice and lysed in 750 μ l MES-buffered saline (MBS: 25 mM MES pH 6.5, 150 mM NaCl), containing 1% Triton X-100, 1 mM Na₃VO₄ and protease inhibitor cocktail (Roche, Indianapolis, IN) for 30 min on ice. Cell lysate was Dounce homogenized, adjusted to 40% sucrose and placed at the bottom of an ultracentrifuge tube. A 5-30% linear sucrose gradient was then placed above the homogenate and the mixture was centrifuged at 45.000 rpm for 16h at 4°C in an SW60 rotor (Beckman Instruments, Palo Alto, CA). Lipid raft fractions are visible as a light-scattering band migrating at approximately 20% sucrose. Twelve 0.375 ml fractions were collected from the top to the bottom of the gradient. The lipid rafts were separated from other fractions, diluted in MBS and centrifuged at 30.000 g for 30 min at 4 °C. Protein content of total membrane and lipid rafts was determined by BCA assay (Pierce Rockford, Ill) using bovine serum albumin as a standard.

Lipid extraction and thin-layer chromatography

Total lipids were extracted according to the method of Folch et al. [31]. Total membranes (50 μ g) and lipid rafts (1.5 μ g) were diluted with equal volume of deionized water. Total lipids were dissolved into chloroform/methanol (2:1 v/v) and applied to TLC plates. The spot corresponding to cholesterol was first identified by co-migration with the cholesterol standard. Cholesterol was separated by TLC using acetic acid: hexane: ether ethyl (1:70:30 v/v/v) as the developing solvent and was visualized by copper acid (3% w/v) staining. The plate was heated for a few minutes at 180°C. The quantity of cholesterol was determined by densitometry and

comparison with 20 μg of standard compound using the imaging densitometer GS -700 (Bio-Rad).

Statistical Analysis

All data were represented as mean \pm SEM and analyzed by one-way ANOVA followed by Bonferroni's Multiple Comparison (Fig. 4,5) or by unpaired t-test analysis (Fig. 3) using with the Prism program (GraphPad). $p < 0.05$ was considered significant.

RESULTS

sCT forms amyloid-like oligomers

We first proceeded to obtain and characterize samples of sCT in an early stage of the aggregation process. For this purpose, sCT (1mM) was dissolved in phosphate buffer (5 mM) and allowed to aggregate for 3 h at room temperature, to facilitate the aggregation process. To obtain sCT monomers, sCT was dissolved in pure deionized distilled water, immediately before use, as previously suggested [14,15,16]. As demonstrated by Energy Filtered-Transmission Electron Microscopy (EF-TEM) analysis, aggregated sCT (Fig.1A) formed small isolated globules of mean diameter of about 14 nm (Fig.1C). These features represent the classical intermediates occurring between monomers and mature fibrils and show a remarkable similarity with A β oligomers and other amyloid proteins [19,20]. No mature fibrils were observed. When sCT was dissolved in distilled water and observed without pre-incubation (Fig.1B), only very small white dots, of mean diameter of about 5 nm (Fig.1C), were present, likely representing sCT monomers or small molecular weight oligomers, in analogy to what observed for bovine [14,15] and human [16] CT. CD data (Fig. 1D), processed according to Whitmore and Wallace [32], indicated that, in sCT freshly dissolved in distilled water, the random coil component was 72% while the β -structure was 23% (5% of α -structures). It is well known that sCT in water solution and in monomeric state is mainly random coil [33]. CD data from sCT dissolved in phosphate buffer and aggregated for 3 h (Figure 1D), show that, in this very early aggregation stage, the amount of β -structure rose to about 28% while the amount of random coil fell to 67%. The amount of α -structures did not change. This is consistent with the aggregation process

described for the amyloid proteins, where random components tend to organize in β -structures. This result is in good agreement with similar experiments [11, 16].

sCTOs damage fine neurites and synapses like A β oligomers

Neuritic damage is a well-described effect of A β in hippocampal neurons in culture. Furthermore, synaptic damage, a hallmark of AD, has been put in relation to the presence of A β oligomers [34]. Since sCTOs have morphological and structural similarities to amyloid proteins, we asked if the effects induced by sCTOs on neurites and synapses in mature hippocampal neurons were similar to those induced by A β oligomers. We immunostained mature neurons for MAP2, a component of the dendritic cytoskeleton, to label the neuritic tree, and for synaptophysin, an integral protein of synaptic vesicles, to label synapses. When compared to control cultures, both sCTOs and A β oligomers induced a remarkable dendritic loss, especially in the finest branches (Fig. 2, upper panel). In addition both of them caused an evident decrease in the number of synaptic boutons (Fig. 2, lower panel).

sCTOs are cytotoxic for mature hippocampal neurons, but not for other cell types

Amyloid oligomers are thought to initiate an apoptotic cascade, leading to neuronal cell death [35,36]. To verify if sCTOs had a pro-apoptotic effect, to more closely link cytotoxicity to Ca²⁺ imbalance, we applied a protocol reminiscent of the “delayed treatment paradigm”, described by Choi in 1988 [37], where a brief (20 min) excitotoxic insult is followed by 24-h-

recovery in cell culture medium. The overstimulation of N-methyl-D-aspartate (NMDA) receptor obtained with this treatment induces an intense increase in intracellular Ca^{2+} that preferentially triggers an apoptotic pathway of cell death. Similarly, we exposed the cell cultures to 20-min treatment with sCTOs and allowed a 24-h-recovery in cell culture medium in absence of sCTOs. We estimated the apoptotic rate by identifying nuclei showing fragmentation or condensation of chromatin, using the Hoechst stain. Mature hippocampal neurons, at DIV14, showed a marked increase of the apoptotic rate, when treated with sCTOs, with respect to controls (Fig.3A,B). The results were confirmed by the Terminal transferase-mediated dUTP-biotin Nick End-Labeling TUNEL assay (Fig.3B,C). We then analyzed sCTOs cytotoxicity in immature hippocampal neurons (6 DIV) and non-neuronal primary cultures (cortical astrocytes from rat embryos) or immortalized cell lines of non-neural histogenesis (MG63 osteoblasts and NIH-3T3 fibroblasts). None of them showed modifications of the apoptotic rate, as shown by the Hoechst stain. Growth curves of MG63 osteoblasts (Fig. 4) and NIH-3T3 fibroblasts (data not shown) were also unaffected. It should be noted that apoptosis in mature hippocampal neurons was related to the presence of oligomeric structures, since when CT was dissolved in water and used without previous incubation, no significant increase in cell death was observed (Fig.5A), as already indicated [13,14].

sCTOs increase intracellular Ca^{2+} in mature hippocampal neurons, but not in other cell types

A number of findings support the hypothesis that amyloid proteins form Ca^{2+} -permeable channels on cell membranes, which induce a perturbation of cytosolic Ca^{2+} [6-8] and trigger apoptosis [38]. We monitored intracytoplasmic free Ca^{2+} by Fura 2AM, after administration of

sCTOs, in mature and immature hippocampal neurons, primary cortical astrocytes, MG63 osteoblasts and NIH-3T3 fibroblasts (Fig.3D,E). While mature hippocampal neurons had an intense and sustained increase in cytosolic Ca^{2+} , which involved all the recorded cells without recovery for the duration of the experiments (8 min) (Fig. 3D,E), immature hippocampal neurons, astrocytes and NIH-3T3 fibroblasts did not show any change in intracellular Ca^{2+} . MG63 osteoblasts did show a perturbation in cytosolic Ca^{2+} , but it consisted in a much lower and rapidly recovering increase, which affected only a minority of cells. As for increased apoptosis, Ca^{2+} perturbation in mature hippocampal neurons was related to the presence of oligomers, since treatment with CT monomers did not induce any change in intracellular Ca^{2+} (Fig. 5B). Thus the pro-apoptotic effect of sCTOs in hippocampal neurons was conceivably related to the intense and unbalanced Ca^{2+} increase, which was lacking in the other cell types.

Lipid rafts are more abundant in mature hippocampal neurons than in the other cell types

We have recently shown that the presence of GM1- and cholesterol-enriched lipid rafts favors the formation of Ca^{2+} -permeable, sCTO ion-channels in model membranes [18]. To quantitatively investigate differences in the lipid composition of the different cell types, we evaluated the ratio between cholesterol in low-density Triton-insoluble (LDTI) plasma membrane domain and total cholesterol (weight %), as a measure of lipid raft content in the plasma cell membrane. Results clearly indicate that mature neurons show the highest content in lipid rafts (Fig.6A), with respect to the other cell types.

Is sCTO-induced Ca^{2+} rise due to preexisting ion channel stimulation?

Some authors sustain that amyloid oligomers insert into the neuronal membrane to form a cation-conducting pore, leading to Ca^{2+} dysregulation, and favor the hypothesis that stimulation of preexisting Ca^{2+} -permeable ion channels is not involved [5,39]. Other authors have, in contrast, evidenced a role for glutamate receptor activation in the neurotoxic effect of amyloid proteins, such as $\text{A}\beta$ [40-42] and HypF-N [43]. To explore the role of glutamate receptors in sCTO neurotoxicity, we first tried to establish if the observed Ca^{2+} increase in mature hippocampal neurons after sCTO treatment was due to an intracellular release or to an extracellular influx. To this aim, we used thapsigargin, a specific sarcoplasmic/endoplasmic reticulum calcium ATPase pump inhibitor, which depletes intracellular Ca^{2+} stores. Two μM thapsigargin evoked only a very inconspicuous rise in intracellular Ca^{2+} , consistent with Ca^{2+} leakage from intracellular stores, but subsequent application of sCTOs oligomers produced a large and rapid increase, similar to that observed in control cells without thapsigargin, suggesting that sCTO-induced Ca^{2+} rise was mostly due to an extracellular influx (Fig. 7A). Then we moved to analyze if the NMDA receptor (which is the glutamate receptor mostly responsible for excitotoxic Ca^{2+} rise in neurons) was involved in the influx. First, we pretreated hippocampal neurons with MK801, a specific NMDA inhibitor. MK801 poorly affected sCTO-driven Ca^{2+} influx (Fig. 7B), causing only a small delay in the response, while it completely blocked NMDA-driven Ca^{2+} increase (data not shown). sCTOs, however, could interact with the NMDA receptor at a site different from that bound to MK801, thus modifying receptor structure and regulation. To this purpose, we pre-treated hippocampal neurons with antibodies against the NR1 subunit, which is a mandatory component of all NMDA receptors, to check if immunoglobulins specifically bound to the receptor could hinder its interaction with sCTOs and suppress Ca^{2+} influx. In the presence of anti-NR1, again the response to sCTOs was slightly delayed; however,

after a few seconds, it reached the same intensity and stability as in absence of the antibody (Fig. 7C). To further analyse sCTO response, we pre-treated mature hippocampal neurons with an antibody-anti GM1, in the attempt to block the pore formation. The presence of the antibody completely abolished sCTOs-induced Ca^{2+} increase. After 10 min, an administration of 100 μM NMDA induced a classical abrupt and intense Ca^{2+} rise, supporting the fact that the NMDA receptor activity had not been modified by the presence of anti-GM1 antibody (Fig. 7D). The response was reliably due to a specific interaction with GM1, since unrelated antibodies, such as anti-BSA, at the same concentration, did not affect sCTO response (Fig. 7E). These results suggest that although NMDA receptor may be rapidly activated at the beginning of the response, the formation of sCTO Ca^{2+} -permeable pores is the main mechanism responsible for Ca^{2+} dysregulation.

Depletion of gangliosides affects sCTO response in mature hippocampal neurons

To further support the hypothesis that the highest content in lipid rafts in mature hippocampal neurons was related to their susceptibility to the sCTO pro-apoptotic effect, we tested if lipid raft depletion modified sCTO-induced cytotoxicity and Ca^{2+} influx. In mature hippocampal neurons treated with neuraminidase (NAA), which specifically removes gangliosides, a characteristic component of lipid rafts, sCTO-driven increase in the apoptotic rate was remarkably reduced (Fig.8 A); in parallel, cytosolic Ca^{2+} increase was inhibited (Fig.8 B). This observation strongly supports the hypothesis that the high content in lipid rafts promotes sCTO neurotoxicity. In addition, we also pre-treated the cells with MCDX, in order to remove cholesterol from the plasma membrane. Again, we observed a significant reduction in the apoptotic rate of hippocampal neurons, although they showed a variable response to sCTOs in

terms of Ca^{2+} influx. The majority of treated cells rapidly reverted to the initial Ca^{2+} level (Fig.8

B).

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DISCUSSION

Pathologies deriving from misfolded amyloid proteins are particularly frequent in CNS. The reasons for this peculiar vulnerability are presently unknown, but several hypotheses may be formulated. The brain may provide a particularly suitable environment for protein misfolding process, or, alternatively, neuronal cells may be particularly sensitive to the toxic potential of amyloid proteins. We used CT, a protein that undergoes a process of amyloid aggregation, to investigate if neuronal cells are more vulnerable to amyloid proteins than cells of different histogenesis, a comparative study that to our knowledge has never been performed. We chose sCT because it is known to aggregate more slowly than human CT [11,16]. To observe the early stages of oligomerization, incubation was limited to 3 h to obtain a sample rich in oligomers, but devoid of fibrils.

Consistently with its amyloid nature, in mature (14 DIV) hippocampal neurons, sCTOs induced cell damage that resembled remarkably that induced by A β , such as altered neuritic tree, with loss of the finest branches, and synaptotoxicity, evidenced by loss or alteration of synaptic structures. This is of particular relevance since damage of synapses is considered to be responsible for neuronal dysfunction underlying AD cognitive impairment and represents the pathologic finding that best correlates with the degree of dementia [4]. To highlight specific susceptibility of neuronal cells to amyloid toxicity, we compared sCTO toxicity in mature and immature (6 DIV) hippocampal neurons to that of two immortalized cell lines (MG63 osteoblasts and NIH-3T3 fibroblasts). In addition, we also analysed the effects on primary astrocytic cultures from rat fetal brain, to exclude that immortalized lines could present a more resistant phenotype, due to their manipulated genome. Our results show that mature, but not immature, hippocampal neurons respond to sCTOs with an evident increase in apoptosis, while the other cell types did

not show any increase in cytotoxicity. Furthermore, sCTO administration to mature, but not immature hippocampal neurons induced an intense and sustained rise in intracellular Ca^{2+} , while the other cell types were nearly unresponsive. MG63 osteoblasts did show a slight response in terms of Ca^{2+} rise, but the increase was much lower than the one observed in neurons, rapidly disappeared and affected a very small proportion of cells.

When considering the pathogenesis of amyloid protein neurotoxicity, two main possibilities are presently considered: i) oligomers of small molecular weight insert into membrane bilayers to create cytotoxic pores in a relatively non-specific mechanism [5,7-9,39]; ii) oligomers bind to particular membrane targets as highly specific ligands [34]. In favour of the first hypothesis is the observation that several disease-related amyloid proteins share a common mechanism for oligomer-mediated toxicity, notwithstanding their different primary sequence [5,39]. According to this theory, the toxicity of amyloid oligomers is intrinsically related to their aggregation state and point to the formation of pores, with consequent dysregulation of intracellular Ca^{2+} , as a primary pathogenetic mechanism.

In our experiments, we found that mature hippocampal neurons were more susceptible to sCTO-induced Ca^{2+} dysregulation, at difference with the other examined cell types. Which characteristic may be at the basis of this peculiar reactivity? A large body of evidence sustains a role for lipid rafts in amyloid-induced neurotoxicity. Lipid rafts have been put in relation to $\text{A}\beta$ formation [22,23]. Of particular interest is their involvement in the mechanisms by which amyloid oligomers interact with neuronal plasma membrane and triggers a neurotoxic response. It has been reported that the neurotoxicity of some amyloid proteins, such as $\text{A}\beta$ peptides and calcitonin, is strongly reduced by the depletion of ganglioside and/or cholesterol [15,24]. In addition, it has been shown that $\text{A}\beta$ binds to sialic acid-containing gangliosides [25,26,44]. The neuronal plasma membrane is particularly rich in lipid rafts. Thus, content in lipid rafts higher

than the other cell types could render neurons more vulnerable to amyloid toxicity. This hypothesis was confirmed by our results, where mature neuronal cells showed a much more elevated content in lipid rafts of the other cells types examined. It has also been demonstrated that lipid rafts increase in the plasma membrane during in vitro maturation in hippocampal neurons [45]. This could explain why mature neurons were sensitive to sCTO toxicity, but immature neurons were not. In agreement with this hypothesis, we found that a much higher content in lipid rafts was present in the plasma membrane of 14 DIV hippocampal neurons, with respect to 6 DIV neurons, primary astrocytic cell cultures, MG63 osteoblasts and NIH-3T3 fibroblasts.

We have recently shown that the presence of GM1- and cholesterol-enriched lipid rafts favors the formation of Ca^{2+} -permeable, sCTO ion-channels in model membranes [18]. The high content in lipid rafts could facilitate the formation of sCTO Ca^{2+} -permeable pores in mature hippocampal neurons, a mechanism hypothesized for other amyloid proteins [39] that implies a nonspecific interaction, independent from the stimulation preexisting Ca^{2+} -permeable plasma membrane ion channels. This view has, however, been challenged by authors who evidenced a role for glutamate receptors in $\text{A}\beta$ [40-42] and HypF-N, the N-terminal domain of the *Escherichia coli* hydrogenase maturation factor, a protein that is not associated with any amyloid disease but shows an aggregative behavior [43]. It has been hypothesized at this regard that oligomers may act at two steps, separated in time [43]. A first step, very rapid (in the order of seconds) where Ca^{2+} increases following glutamate receptor stimulation by the oligomers, followed by a second, delayed step where oligomers permeabilize nonspecifically the cell membrane, possibly via the formation of pores. Our results show that sCTO-induced Ca^{2+} entry was poorly affected by the presence of MK801, an NMDA receptor blocker, or pretreatment with an anti-NR1 antibody, used to mask possible sites of interactions between sCTOs and the

NMDA receptor, the responses in these cases being only slightly delayed. On the contrary, pre-treatment of neurons with an antibody against GM1 completely suppressed sCTO-driven Ca^{2+} rise, without altering NMDA receptor activity. On the basis of the delayed Ca^{2+} rise observed when NMDA receptor was blocked, we cannot exclude that a component due to an interaction between sCTOs and the NMDA receptor is present, or that the AMPA type of glutamate receptors, which have not been examined in the present study, may be involved. However, the block of Ca^{2+} response obtained with an anti-GM1 antibodies strongly supports the conclusion that the intense and protracted Ca^{2+} dysregulation observed after sCTOs treatment is reliably due to the pore formation. As further support to the role of lipid rafts in sCTO toxicity, we observed that lipid raft disruption obtained by NAA, which removes sialic acid from gangliosides, a characteristic component of lipid rafts, inhibited Ca^{2+} rise and protected against sCTO neurotoxicity, probably modifying the plasma membrane area susceptible to the insertion of the pore-like structures. This perspective is, in our opinion, pretty innovative, since it does not focus on the inhibition of the amyloid oligomer formation but to the hindrance of its interaction with the neuronal cell membrane. MCDX, which removes cholesterol, also reduced both sCTO toxicity and Ca^{2+} rise, but with a less clear-cut effect than NAA. Actually, the role of cholesterol in amyloid toxicity is quite controversial. A comparative study on the susceptibility of different cell lines to toxicity of the amyloid protein HypF-N showed inverse relation to membrane content in total cholesterol [46]. On the other hand, it has recently been demonstrated that membrane cholesterol levels in mature neuron are significantly higher than those detected in young ones and that decreasing membrane cholesterol in mature neurons reduces their susceptibility to $\text{A}\beta$ -induced cell death [47].

The present findings show that our previous results on the mechanism of Ca^{2+} -permeabilization induced by sCTOs in model membranes also applies to plasma membrane of

living cells, and that sCTOs behaves as a pathologic amyloid protein inducing a pro-apoptotic, neurotoxic effect. This response is likely caused by intracellular Ca^{2+} dysregulation, accompanied by neuritic and synaptic changes similar to those observed after in vitro $\text{A}\beta$ treatment and linked to a particular membrane lipid composition: the coexistence of gangliosides and cholesterol, characteristic of lipid rafts. In conclusion, we suggest that the high content in lipid rafts of mature neuronal plasma membrane may underlie a particular susceptibility to the cytotoxic effect of amyloid protein. This characteristic may contribute to the specific vulnerability of CNS to misfolded protein diseases and could be the object of further studies aimed at defining new targets for innovative therapeutic strategies.

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ACCEPTED MANUSCRIPT

FIGURE LEGENDS

Fig. 1. sCT forms typical amyloid aggregates in PBS. EF-TEM revealed that 1 mM sCT formed small globular oligomers after 3h of incubation in phosphate buffer at room temperature (A), of a mean diameter of about 14 nm (C). Mature fibrils were not present. sCT dissolved in distilled water (B) and analyzed without incubation showed that only very small white dots could be observed, likely representing monomers or small molecular weight oligomers, of mean diameter of about 5 nm (C). D: The CD spectra are reported in D. sCTO (dotted line) gave 67% of random coil, 28% of β -structure and 5% of α -structure, while sCT monomers (continuous line) gave 72% for the random component, 23% for the β -structure and 5% of α -structure. Bar=50 nm.

Fig. 2. sCTOs and A β oligomers similarly damage the neuritic tree and synapses in mature hippocampal neurons. Mature hippocampal neurons were treated with 80 μ M sCTOs or 20 μ M A β for 20min, washed, fed with conditioned medium and kept in culture for 24h. After fixation, cells were immunostained for MAP2 (red) and synaptophysin (green). Nuclei were counterstained with Hoechst 33258 (blue). In control cultures, anti-MAP2 antibody evidences a rich dendritic tree (upper panel, bar=50 μ m), while synapses appear as green dots along the dendrite (lower panel, bar=10 μ m). After sCTO or A β treatments, the extension of the dendritic tree was evidently reduced (upper panel) and the number of synapses decreased (lower panel).

Fig. 3. sCTO induces increase in apoptosis and intracellular Ca²⁺ levels in mature hippocampal neurons. A: Mature and immature hippocampal neurons, primary astrocytes, 3T3 fibroblasts and MG3 osteoblasts were treated with sCTO for 20min, fed with conditioned medium and kept in culture for further 24h. After fixation, cells were stained with Hoechst 33258 and apoptotic

nuclei counted. Apoptotic nuclei are expressed as percentage of total nuclei. Bars represent the mean \pm S.E.M. of at least six independent experiments. A significant increase in the apoptotic rate was evident only for mature neurons while all other cell types did not show any difference in cell death, compared to their control cultures. B: Details of apoptotic nuclei stained with Hoechst (arrowheads) and TUNEL. C: After treatment with sCTOs, as already described, mature hippocampal neurons were fixed and treated for the TUNEL assay. Positive nuclei were counted and percentage of apoptotic nuclei is expressed as mean \pm S.E.M. from four different experiments in duplicate. TUNEL assay confirmed that apoptosis more than doubled in sCTO-treated mature hippocampal neurons. D: Representative experiments of the effects of sCTOs on intracellular Ca^{2+} by optical fluorimetric recordings with Fura-2AM in the different cell cultures. Fura-2AM-loaded cells were recorded during baseline and after application of sCTOs (arrows). Each line corresponds to one cell. In mature hippocampal neurons only, sCTOs induced a rapid and intense elevation of intracellular Ca^{2+} . E: Intracellular Ca^{2+} values were compared before (-60s) and after the application of sCTOs (480s), for each different cell culture. Bars represent mean values \pm S.E.M of Ca^{2+} levels of Figure D. At least 7 cells were evaluated in each experiment. A significant increase in intracellular Ca^{2+} was observed after treatment with sCTOs only in mature hippocampal neurons. Bars=50 μm . $^* = p < 0.05$ vs control

Fig. 4 sCTO does not influence cell proliferation. MG-63 osteoblasts were treated with 25, 60, 80 or 120 μM sCTO for 20 min and then let grow in the medium in absence of sCTO for 24, 48 and 72 h. Cells were then detached and counted. Results are represented as mean \pm S.E.M. of three different experiments. No modifications of cell growth were found in treated cells with respect to control.

Fig. 5. sCTO neurotoxicity is related to the presence of oligomers. A: Mature hippocampal neurons were treated sCTOs or sCT freshly dissolved in water (sCT monomers), as already described. After fixation, cells were stained with Hoechst and apoptotic nuclei counted. Apoptotic nuclei are expressed as percentage of total nuclei. Bars represent the mean \pm S.E.M. of at least six independent experiments. sCT dissolved in water did not induce a significant increase in apoptosis. B: Representative experiments of the effects of treatment with sCT monomers or sCTOs on intracellular Ca^{2+} by optical fluorimetric recordings with Fura-2AM in mature hippocampal neurons. Fura-2AM-loaded cells were recorded during baseline and after application of sCT monomers (first arrow) and sCTOs (second arrow). Each line corresponds to one cell. A rapid and intense elevation of intracellular Ca^{2+} was obtained after sCTOs, but not after sCT monomers. $*=p<0.05$

Fig. 6 Mature neurons have the highest content in lipid rafts. To quantitatively investigate differences in the content in lipid rafts of the various cell types used in our experiments, we evaluated the weight ratio between LDTI and total cholesterol. Results indicate that the plasma membrane of mature hippocampal neurons showed a much higher content in lipid rafts than immature neurons and the other cell types.

Fig. 7 Stimulation of NMDA receptors does not play an important role in sCTO-driven Ca^{2+} influx. Changes in intracellular Ca^{2+} were evaluated by optical fluorimetric recordings with Fura-2AM in mature hippocampal neurons. Each line corresponds to one cell. To establish if sCTO-induced Ca^{2+} increase in mature hippocampal neurons was due to an intracellular release or to an extracellular influx, possibly mediated by glutamate receptors, mature hippocampal neurons were treated with 2 μM thapsigargin, which depletes intracellular Ca^{2+} stores. Thapsigargin

evoked an extremely small rise in intracellular Ca^{2+} , consistent with Ca^{2+} leakage from intracellular stores, while sCTOs, administered after a few minutes, produced a much more rapid and intense increase, similar to that observed in control cells, suggesting that sCTO-induced Ca^{2+} rise was mostly due to an extracellular Ca^{2+} influx (Fig. 7A). To analyze if the NMDA receptor (which is the glutamate receptor mostly responsible for excitotoxic Ca^{2+} rise in neurons) was involved in the influx, we pretreated hippocampal neurons with MK801, a specific NMDA inhibitor. MK801 did not affect sCTO-driven Ca^{2+} influx (Fig. 7B), while it blocked NMDA-driven Ca^{2+} increase (data not shown). To rule out the hypothesis that sCTOs could bind to the NMDA receptor at a site different from that blocked by MK801, we pre-treated hippocampal neurons with antibodies against the NR1 subunit, to mask possible sites of interaction between sCTOs and the receptor. Even in the presence of anti-NR1, after a slight delay, the response to sCTOs reached the same intensity and stability as in absence of the antibody (Fig. 7C). On the contrary, pre-treatment with an antibody-anti GM1, aimed at blocking the pore formation, did abolish sCTOs-induced Ca^{2+} increase. After 10 min, an administration of 100 μM NMDA induced a classical abrupt and intense Ca^{2+} rise, supporting the fact that the NMDA receptor activity had not been modified by the presence of anti-GM1 antibody (Fig. 7D). Pre-treatment with anti-BSA IgGs, an unrelated antibody, did not affect sCTO response (Fig. 7E).

Fig. 8. Lipid raft depletion in the neuronal plasma membrane affects sCTO-induced neurotoxicity and Ca^{2+} perturbation. . A: Mature hippocampal neurons were given sCTOs as already described, with or without pretreatment with MCDX or NAA (the effect of MCDX or NAA alone is also shown). Apoptotic nuclei are expressed as percentage of total nuclei. Bars represent the mean \pm S.E.M. of at least six independent experiments. B: Representative experiments of the effects of MCDX or NAA pretreatment on intracellular Ca^{2+} perturbation

induced by sCTOs. Fura-2AM-loaded cells were recorded during baseline and after application of sCTOs in control cultures or cultures pretreated with MCDX or NAA. Each line corresponds to one cell. Intracellular Ca^{2+} showed an initial and mostly transitory rise in cells treated with MCDX, while it was completely unaffected by sCTOs in all cells pretreated with NAA.

*= $p < 0.05$

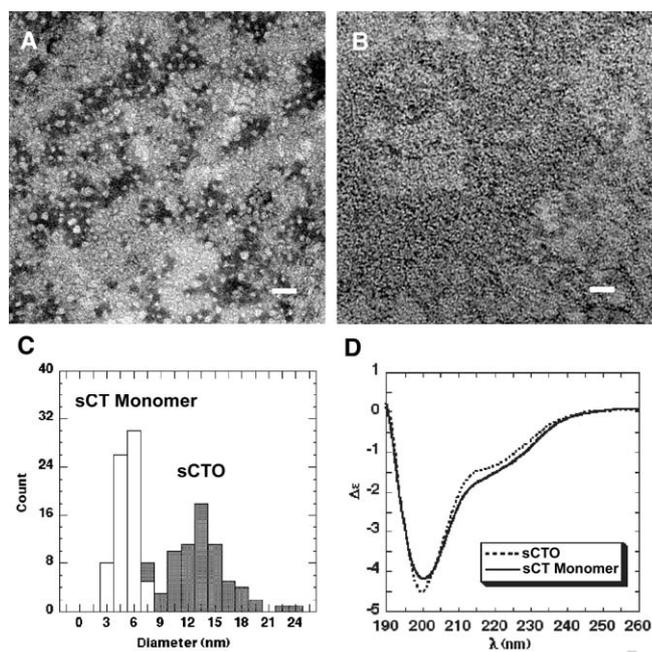


Fig. 1

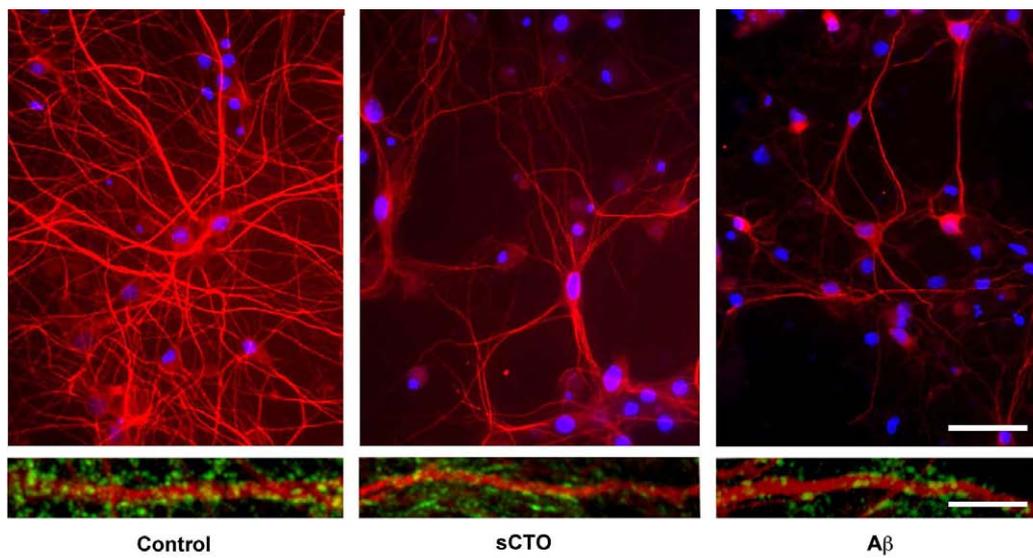


Fig. 2

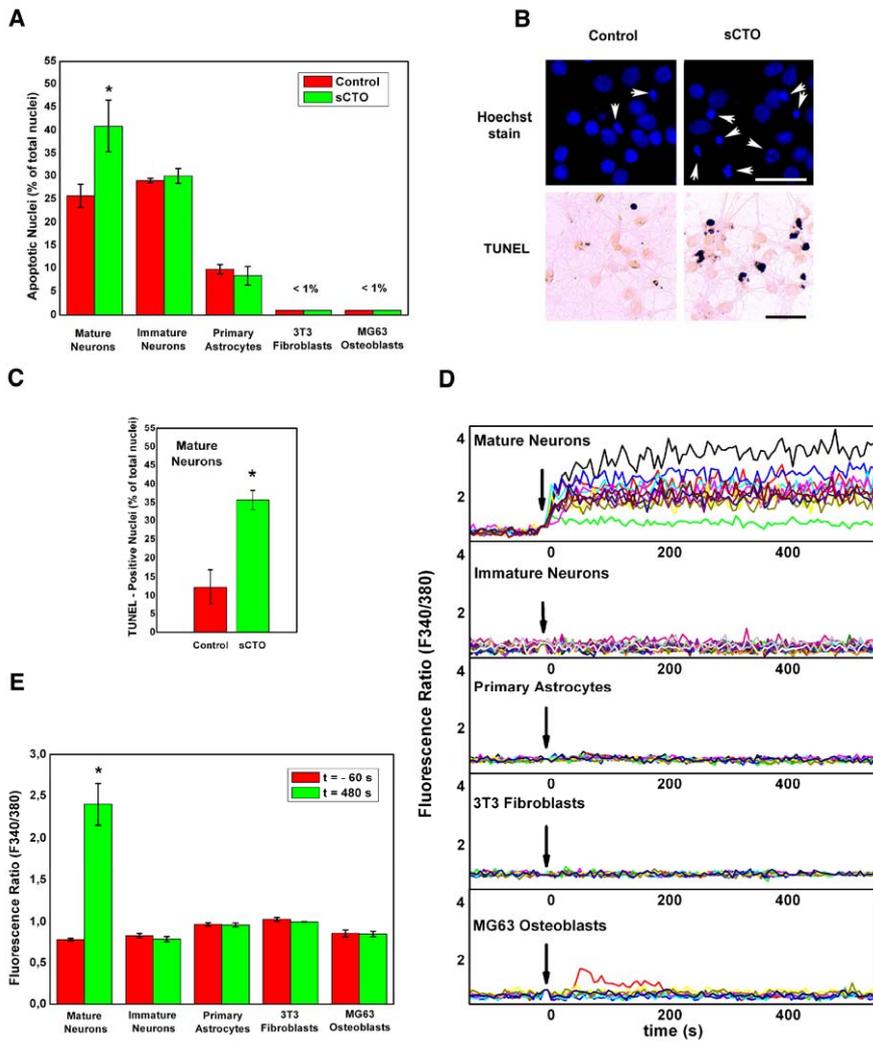


Fig. 3

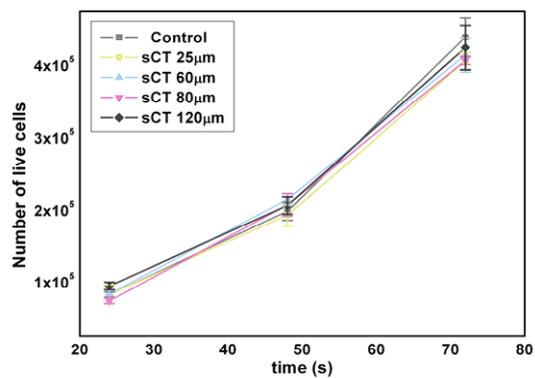


Fig. 4

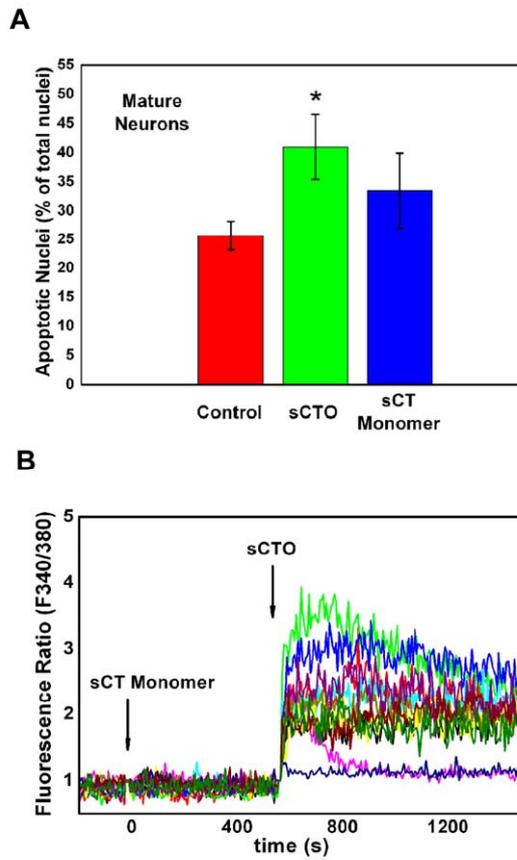


Fig. 5

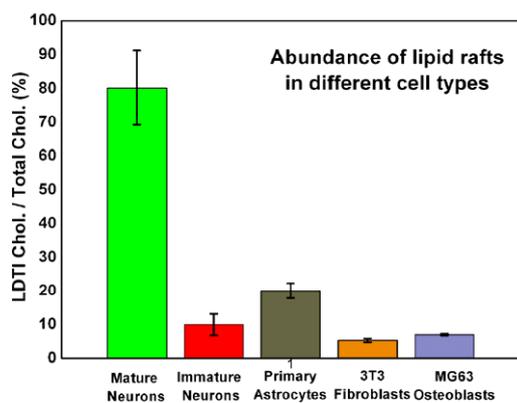


Fig. 6

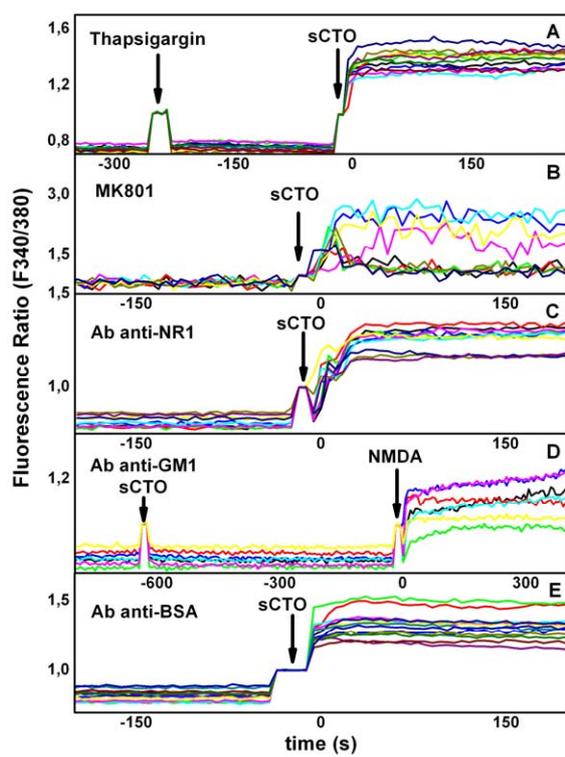


Fig. 7

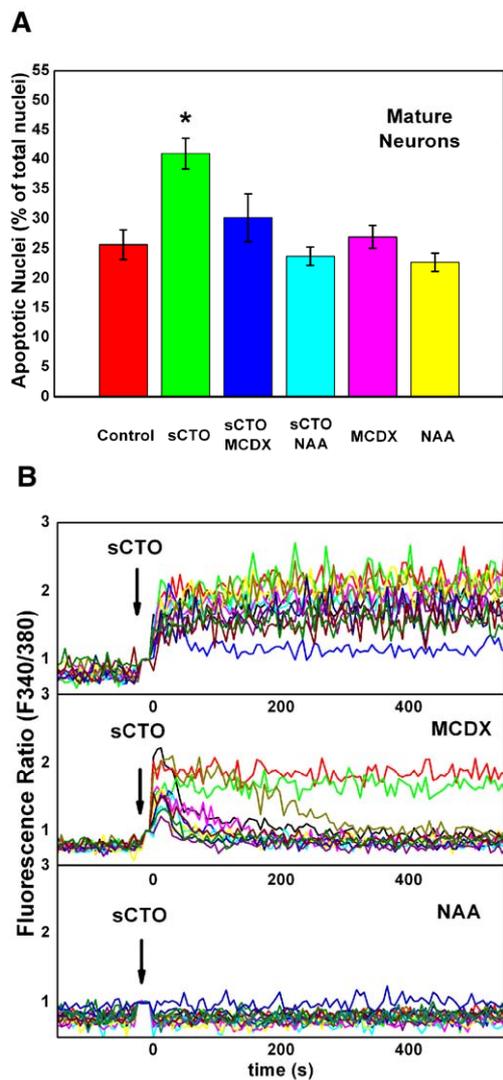


Fig. 8