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Amyloid β oligomers induce Ca^{2+} dysregulation and neuronal death through activation of ionotropic glutamate receptors

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Summary

Amyloid beta (Aβ) oligomers accumulate in brain tissue of Alzheimer disease patients and are related to pathogenesis. The precise mechanisms by which Aβ oligomers cause neurotoxicity remain unresolved. In this study, we investigated the role of ionotropic glutamate receptors on the intracellular Ca^{2+} overload caused by Aβ. Using rat cortical neurons in culture and entorhinal-hippocampal organotypic slices, we found that Aβ oligomers significantly induced inward currents, intracellular Ca^{2+} increases and apoptotic cell death through a mechanism requiring NMDA and AMPA receptor activation. The massive entry of Ca^{2+} through NMDA and AMPA receptors induced by Aβ oligomers caused mitochondrial dysfunction as indicated by mitochondrial Ca^{2+} overload, oxidative stress and mitochondrial membrane depolarization. Importantly, chronic treatment with nanomolar concentration of Aβ oligomers also induced NMDA- and AMPA-receptor dependent-cell death in entorhinal cortex and hippocampal slice cultures. Together, these results indicate that overactivation of NMDA and AMPA receptor, mitochondrial Ca^{2+} overload and mitochondrial damage underlie the neurotoxicity induced by Aβ oligomers. Hence, drugs that modulate these events can prevent from Aβ damage to neurons in Alzheimer´s disease.

Introduction

The molecular mechanisms responsible for the development of the idiopathic cases of Alzheimer disease (AD) are unknown. Growing evidence indicates that cerebral elevation and accumulation of Aβ peptide mediate many aspects of AD pathogenesis. It has been difficult to determine which of the forms of Aβ induces neuropathological changes that characterize the disease. Oligomers have been found in mouse models of AD (Billings et al., 2005; Oddo et al., 2006) and have been detected in CSF (Kuo et al., 1996; Kayed et al., 2003) and brain tissue (Gong et al., 2003; Kayed et al., 2003; Lacor et al., 2004) of AD patients, where the soluble Aβ species appear to correlate with disease progression (Lue et al., 1999; McLean et al., 1999; Naslund et al., 2000). Several mechanisms have been proposed to understand the Aβ-mediated neurodegeneration. AD has been related to a general dyshomeostasis of $[Ca^{2+}]_i$. This hypothesis is substantiated by reports on dysregulation of $[Ca^{2+}]_i$ promoted by Aβ (LaFerla, 2002). Whether Aβ directly alters lipid bilayer by forming pores or acts through proteinaceous receptor, for example α7-nACh or NMDA receptor is a matter of intense debate (Wang et al., 2000, Arispe et al., 2007, De Felice et al., 2007). Growing evidences point to a major role for changes in $[Ca^{2+}]_i$ and Aβ-induced neuronal cell damage. First, Aβ oligomers provoke neurotoxicity by mechanisms that involve a channel independent disruption of the integrity of both plasma and intracellular membranes with elevation in $[Ca^{2+}]_i$ (Demuro et al., 2005). Later, it has been reported that dynamin-1 degradation is the result of calpain activation induced by the Ca^{2+} influx mediated by NMDA receptors in hippocampal neurons (Kelly and Ferreira, 2006). Most recently, some studies have shown that Aβ oligomers bind to or in close proximity to NMDA receptor, triggering neuronal damage through NMDA receptor dependent Ca^{2+}

flux or implicating excitatory receptor activity in oligomer formation and accumulation at synapses (De Felice et al., 2007; Deshpande et al., 2009).

On the other hand, accumulated evidence indicates that physiological concentrations of Aβ peptides can regulate the release of glutamate by acting on glutamatergic terminals (Kabogo et al., 2008). Consistent with that idea, soluble Aβ perturbs synaptic plasticity by altering glutamate recycling at the synapse and promoting synapse depression (Li et al., 2009). Furthermore, previous results have indicated that Aβ toxicity is mediated by Ca^{2+}-dependent glutamate excitotoxicity (Mattson et al., 1992).

Because the link between the Ca^{2+} increase and Aβ toxicity is still controversial, we examined the contribution of various routes of Ca^{2+} entry to Ca^{2+} overload in Aβ-produced neurotoxicity. We found that Aβ oligomers generate glutamate-independent inward currents, dysregulate Ca^{2+} homeostasis and induce cell death through both NMDA and AMPA receptors in cultured neurons or in enthorinal cortex-hippocampus organotypic slices. In this apoptotic neuronal death caspase-dependent and independent pathways are involved. Aβ-induced changes of $[Ca^{2+}]_i$ provoke mitochondrial Ca^{2+} overload, mitochondrial membrane depolarization and ROS generation. Antagonists of NMDA and AMPA receptor reduce deleterious events in mitochondria, indicating that Glu receptors initiate the Aβ-induced Ca^{2+} dysregulation in neurons.

Materials and Methods

Drugs and culture medium. Neurobasal medium, B27 supplement, foetal bovine serum, horse serum and other culture reagents were from Gibco (Invitrogen, Barcelona, Spain). Receptor antagonists MK801, DAP5 (AP5), and memantine were all obtained from Tocris (Cookson, Bristol, UK). CNQX and other chemicals were from Sigma (St Louis, MO, USA). The general caspase inhibitor ZVAD and the caspase-3 specific inhibitor DEVD were purchased in Peptides International (Louisville, KY, USA). DPQ, the PARP-1 inbibitor, was obtained from Calbiochem (La Jolla, CA, USA).

Preparation of Aβ oligomers. Aβ1-42 oligomers were prepared as reported previously (Klein, 2002). Briefly, Aβ1-42 (ABX, Radeberg, Germany) was initially dissolved to 1 mM in hexafluoroisopropanol (Sigma; St Louis, MO, USA) and separated into aliquots in sterile microcentrifuge tubes. Hexafluoroisopropanol was totally removed under vacuum in a speed vac system and the peptide film was stored dessicated at -80 ºC. For the aggregation protocol, the peptide was first resuspended in dry DMSO (Sigma; St Louis, MO, USA) to a concentration of 5 mM and Hams F-12 (PromoCell, Labclinics, Barcelona, Spain) was added to bring the peptide to a final concentration of 100 μM and incubated at 4 ºC for 24h. The preparation was then centrifuged at 14,000 x g for 10 min at 4 ºC to remove insoluble aggregates and the supernatants containing soluble Aβ1-42 was transferred to clean tubes and stored at 4 ºC.

Cortical cell culture. Primary neuron cultures were obtained from the cortical lobes of E18 Sprague-Dawley rat embryos according to previously described procedures (Gottlieb et al., 2006). Cells were resuspended in B27 Neurobasal medium plus 10% FBS (Sigma; St Louis, MO, USA) and then seeded onto poly-l-ornithine-coated glass coverslips at 1 x 10^5 cells per coverslip (12 mm in diameter) and 48-well plates at 1.5 x 10^5 per well. One day later, the medium was replaced by serum-free-, B27-

supplemented Neurobasal medium. The cultures were essentially free of astrocytes and microglia; they were maintained at 37 ºC and 5% CO_2. Cultures were used 8-10 days after plating.

Preparation of organotypic cultures. Brain was removed and two hemispheres were separated in HBSS. Talamus and midbrain was removed and each hemisphere sliced with a tissue chopper (McIlwan Tissue Chopper, Campden Instruments Ltd, Lafayette, IN, USA) in order to obtain coronal slices of 400 µm of thickness. Enthorinal cortex, in connection with hippocampus, were isolated under a dissection microscope, 2 slices plated on each Millicell CM culture inserts (Millipore Ibérica, Madrid, Spain) and maintained in Neurobasal medium supplemented with 0.5% B27, 25% horse serum, 25% HBSS and 25 mg/ml gentamycin at 37°C. Experiments were performed at 7-10 days in vitro. Cultures were immunostained with antibodies to neurofilament-L (NFL 1:200; Cell Signaling Technology, Boston; MA) and choline acetyltransferase (ChAT 40 µg/ml; Chemicon, Millipore Ibérica, Spain) and labeling was revealed with fluorescent goat anti-rabbit and donkey anti-goat secondary antibodies respectively (Alexa Fluor 488, Invitrogen, Barcelona, Spain)

Electrophysiology. Whole-cell recordings were performed at room temperature using the EPC-7 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany). Currents were recorded at a holding membrane potential of -70 mV. Extracellular bath solution with a pH of 7.3 contained the following (in mM): 140 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 mM glucose. Patch-clamp pipettes (3-5 MΩ) were filled with internal solution at a pH of 7.3 containing the following (in mM): 140 K-gluconate, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 EGTA, 2 Mg-ATP, 0.2 Na-GTP.

Ca^{2+} imaging in the cytosol. $[Ca^{2+}]_i$ was determined according to the method described previously (Grynkiewicz et al., 1985). Neurons were loaded with fura-2 AM (5 µM;

Invitrogen, Barcelona, Spain) in culture medium for 30 min at 37ºC. Cells were washed in HBSS containing 20 mM HEPES, pH 7.4, 10 mM glucose, and 2 mM $CaCl_2$ (incubation buffer) for 10 min at room temperature. Experiments were performed a coverslip chamber continuously perfused with incubation buffer at 4 ml/min. The perfusion chamber was mounted on the stage of a inverted epifluorescence microscope (Zeiss Axiovert 35; Oberkochen, Germany) equipped with a 150 W xenon lamp Polychrome IV (T.I.L.L. Photonics, Martinsried, Germany) and a Plan Neofluar 40 x oil immersion objective (Zeiss). Cells were visualized with a high-resolution digital black/white CCD camera (ORCA C4742-80-12 AG; Hamamatsu Photonics Iberica). $[Ca^{2+}]_i$ was estimated by the 340/380 ratio method, using a Kd value of 224 nM. At the end of the assay, in situ calibration was performed with the successive addition of 10 mM ionomycin and 2 M Tris/50 mM EGTA, pH 8.5. Data were analyzed with Excel (Microsoft, Seattle, WA, USA) and Prism (GraphPad Software, San Diego, CA, USA) software.

Measurement of mitochondrial Ca^{2+}. Rhod-2 AM was used to measure mitochondrial Ca^{2+} according to the previous procedure with modifications (Hajnóczky et al. 1995). Rhod2-AM has a net positive charge, which facilitates its sequestration into mitochondria due to membrane potential-driven uptake. Cell were loaded with 2 μM rhod2-AM for 6 hours. The residual cytosolic fraction of the dye was eliminated when the cells were kept in culture for an additional 18h after loading, whereas the mitochondrial dye fluorescence was maintained. Fluorescence images of rhod-2 were acquired using 550 nm excitation and 590 nm emission. The fluorescence of rhod-2 was not calibrated in terms of $[Ca^{2+}]$mit since it is not a ratiometric dye.

Measurement of Ca^{2+} *in organotypic slices.* Ca^{2+} levels in hippocampus were monitored by fluorescence microscopy using the Ca^{2+} indicator fluo-4 (Molecular Probes, Eugene,

OR). Slices were incubated with fuo-4-AM (5 μM and 0.01 % of pluronic acid) for 45 min at 37 ºC. Experiments were performed in a chamber continuously perfused with incubation buffer. Drugs were perfused by a micromanifold with a quartz/polymide tube of 200 μm ID, attached to a micromanipulator in order to perfuse the drugs directly to the cells. The perfusion chamber was mounted on the stage of a Leica DMLFSA up-right microscope equipped with a 150 W xenon lamp Polychrome V (T.I.L.L. Photonics, Martinsried, Germany) and a HCX Apo 40 x water immersion objective (Leica). Cells were visualized with a CCD camera (EM CCD 9100; Hamamatsu Photonics Iberica, Barcelona, Spain).

Cell viability and toxicity assays. Cortical neurons at 8-10 days in culture were exposed for 24 hours to Aβ oligomers. Antagonists and inhibitors were added to the cultures 30 min before the Aβ. Twenty four hours after drug application, cellular damage was estimated by measuring the level of lactate dehydrogenase released (LDH; Cytotox 96®, Promega, Madison, WI) from damaged cells into the culture media. Data were normalized to the activity of LDH released from vehicle-treated cells (100%) and calculated as a percentage of the control. Results were expressed as the means ± SEM of at least three independent experiments performed in triplicates.

Hippocampus-entorhinal cultures were exposed to Aβ oligomers at 100 nM for 4 days. Antagonists were added to cultures 30 min before the Aβ. Cell death in organotypic cultures was evaluated by cellular uptake of propidium iodide (PI). Slices were stained by adding 10 μM PI into the culture for 2 h at 37° C and washed with PBS by two times for 10 min. Slices were fixed with 4% PFA in PBS for 40 min at room temperature (Pozzo-Miller et al., 1994). Afterwards, the slices were excited with 510–560 nm light and the emitted fluorescence acquired at 610 nm using a rhodamine filter on an inverted fluorescence microscope (Cell Observer Z1, Zeiss). PI fluorescence images were

captured with a Plan NeoFluar 2.5 x objective (Zeiss), using an EM CCD camera (Hamamatsu, C9100-13), controlled by Axio Vision program (Zeiss). Images were analyzed with the ImageJ analysis program (NIH, MD, USA) and neuronal cell death was expressed as the percentage of the mean gray value of each treatment vs. control.

Measurement of intracellular reactive oxygen species. Neurons were exposed to Aβ oligomer alone or with GluR antagonists as described. Cells were loaded with CM-H2DCFDA at 30 μM to assay the ROS levels. Calcein-AM (1 μM; Molecular Probes, Invitrogen, Barcelona, Spain) was used to quantify the number of cells within the reading field. Fluorescence was measured using a Synergy-HT fluorimeter (Bio-Tek Instruments Incl, Beverly, MA, USA) and excitation and emission wavelengths for CM-H2DCFDA and calcein were as suggested by the supplier. All experiments (n≥3) were performed at least in quadruplicate and plotted as means ± SEM.

Analysis of mitochondrial membrane potential. Neurons were exposed to Aβ oligomers alone or in presence of different drugs and the changes in mitochondrial membrane potential were monitored by reduction of JC-1 (Molecular Probes, Invitrogen, Barcelona, Spain), according to the manufacturer protocol. Briefly, after drug treatment, cells were loaded with 3 μM JC-1 for 15 min at 37 ºC and were washed with HBSS without phenol red by two times to eliminate the excess of dye. In the cytosol the monomeric form of this dye fluoresces green (excitation at 485 nm, emission at 527 nm), whereas within the mitochondrial matrix highly concentrated JC-1 forms aggregates that fluoresce red (excitation at 485 nm, emission at 590 nm). Both JC-1 monomers and aggregates were detectable using a Synergy-HT fluorimeter (Biotek Instruments) and the changes in mitochondrial potential were calculated as the red/green ratio in each condition. All experiments ($n \geq 3$) were performed at least in triplicate and plotted as mean ± SEM.

Statistical Analysis. Data are presented as means ± SEM. Statistical analysis was carried out with the Student *t* test and, in all instances, a value of $p < 0.05$ was considered significant.

Results

Aβ oligomers activate NMDA and AMPA receptors in neurons

Dysregulation of intracellular Ca^{2+} homeostasis may underlie Aβ peptide toxicity in AD but the mechanisms are unknown. Here, we tested whether the activation of ionotropic GluRs is involved in neuronal response to Aβ 1-42 oligomeric peptide. Previous studies have shown that Aβ oligomers produce reversible synapse loss (Shankar et al., 2007), neuronal oxidative stress (De Felice at al., 2007) and dynamin-1 degradation (Kelly and Ferreira, 2006) through an NMDA receptor dependent mechanism. First, using electrophysiological recording and Ca^{2+} imaging methods, we studied whether soluble Aβ oligomers interact with NMDA receptor in neurons. Whole-cell patch-clamp recording was used to examine the effects of Aβ oligomers on cortical neurons in culture. In conditions which favour NMDA receptor activation (glycine 100 μM and no Mg^{2+}), Aβ oligomers (1 μM) induced an inward current in the majority of neurons examined (816 ± 101 pA; n=30). AP5 (100 μM), a competitive NMDA antagonist, reduced the peak of the currents (n=7). Silencing neural activity with TTX (1 μM) or using a calcium-free extracellular bath solution did not attenuate the response to Aβ oligomers suggesting a direct interaction of the peptide with NMDA receptors (n= 11 and 13 respectively; Fig 1A).

To characterize further the properties of Aβ responses, we next monitored the concentration of intracellular Ca^{2+} $[Ca^{2+}]_i$ in cultured cortical neurons. We found that oligomeric Aβ (5 μM) caused a robust and sustained increase in $[Ca^{2+}]_i$ (270 nM, n=42), which is nearly abolished by NMDA receptor antagonists AP5, MK801 and memantine (n=70). Similarly, Aβ applied in Ca^{2+}-free extracellular buffer containing EGTA 50 μM greatly reduced $[Ca^{2+}]_i$ rise (n=11). In contrast, the AMPA/kainate antagonist CNQX

and the voltage-gated Ca^{2+} channel inhibitors nifedipine and verapamil were ineffective (n=66; Fig 1B).

Together these results provide evidence indicating that Aβ oligomers activate NMDA receptors in cortical cultured neurons.

Previous results have shown that Aβ(1-42) but not Aβ(1-40) closely interacts with synaptic AMPA receptors (Parameshwaran et al., 2007). Because of that, we next examined whether Aβ oligomers also activate AMPA receptors in cultured neurons. In the presence of Mg^{2+} and no glycine, Aβ (1 μM) alone elicited a small current (43 ± 14 pA, n= 6) and co-applied with cyclothiazide (CTZ), an inhibitor of AMPA receptor desensitization, activated a sustained current (n=18; Fig 2A) that was blocked by the AMPA/kainate antagonist CNQX (n= 6; Fig 2A). We then measured the effects of antagonists of NMDA or AMPA receptor on $[Ca^{2+}]_i$ in neurons exposed to Aβ plus CTZ (5 μM and 100 μM, respectively; Fig 2B). In these experimental conditions, Aβ oligomers increased the $[Ca^{2+}]_i$ in 308 ± 44.7 nM (100 % of Ca^{2+} increase, control value, n=23) a level that was substantially reduced by CNQX 30 μM (50 ± 4 % of control, n=17) and by AP5 100 μM (45 ± 2 % of control, n=17; Fig. 2B). Co-incubation with CNQX and AP5 further reduced $[Ca^{2+}]_i$ to 14 ± 2 % of control (n=18; Fig. 2B), Any facilitatory role of AMPA receptors on Aβ-induced NMDA receptor response was excluded since in physiological conditions (2 mM Mg^{2+}, 0.1 mM Gly) CNQX did not modify the Aβ-produced inward current (98.3 ± 6.8 % Aβ + CNQX vs 100 % Aβ alone, n=6). These results indicate that Aβ oligomers in presence of CTZ activate AMPA receptors and that the contribution of both receptors to Ca^{2+} influx is substantial.

Aβ oligomers trigger apoptotic neuronal death through NMDA and AMPA receptors

As illustrated above, NMDA and AMPA receptor activation by Aβ oligomers in neurons causes an increase in basal $[Ca^{2+}]_i$ levels. Because GluRs mediate Ca^{2+}-dependent–neuronal cell death, we tested whether activation of these receptors by Aβ is toxic to neurons. Incubation of Aβ oligomers for 24h caused neuronal death in a dose-dependent manner (Fig 3A). In turn, Aβ toxicity (5 μM; 15.2 ±1.8 % cell death vs. non-treated cells) was greatly attenuated by NMDA receptor antagonists AP5 100 μM, MK801 50 μM and memantine 50 μM and by AMPA/kainate receptor antagonist CNQX 30 μM (Fig. 3B).

Previous reports have shown that oligomeric Aβ peptides induce mitochondrial dependent-apoptotic cell death in cultured neurons (Sanz-Blasco et al., 2008). Damage to mitochondria can result in both caspase-dependent and -independent cell death. To characterize whether these two types of apoptosis are involved in GluR-mediated Aβ toxicity, we used the pan-caspase inhibitor ZVAD-F, a caspase-3 inhibitor Ac-DEVD-F and the nuclear enzyme poly (ADP-ribose) polymerase-1 (PARP-1) inhibitor DPQ. We observed that ZVAD-F (50 μM), DEVD (100 μM) and DPQ (30 μM) reduced Aβ-induced cell death (Fig. 3C). Aβ toxicity decreased further by blocking simultaneously caspase-3 and PARP-1 with DEVD + DPQ (Fig. 3C). These results indicate that Aβ oligomers induce caspase-3- and PARP-1-dependent neuronal death via activation of NMDA and AMPA receptors.

Aβ oligomers induce mitochondrial damage by activating glutamate receptors

To characterize further the signalling cascades triggered by Aβ oligomers, we next studied mitochondrial Ca^{2+} uptake in neurons with rhod-2 AM, a cationic indicator which loads selectively into respiring mitochondria. To assess that feature, we used FCCP 25 μM, a protonophore that collapses the mitochondria membrane potential and releases Ca^{2+} from mitochondria. As expected, FCCP induced a rapid and transitory reduction of rhod-2 fluorescence (Fig 4A). In these loading conditions, we assayed the effects of Aβ oligomers on $[Ca^{2+}]_{mit}$. Aβ 5 μM increased $[Ca^{2+}]_{mit}$ levels with respect to the control assay (incubation buffer). Ca^{2+} rise was reduced by NMDA antagonist MK801, AP5 and memantine, whereas CNQX was ineffective (Fig 4B and C). These results indicate that Aβ induces mitochondrial Ca^{2+} overload and that this effect can be prevented by NMDA receptors antagonists.

Aβ induces mitochondrial dysfunction by an NMDA receptor-dependent mechanism in hippocampal neurons (De Felice et al., 2007). Consistently, we observed that NMDA antagonists reduce Aβ-generated oxidative stress (Fig. 4D) and mitochondrial depolarization (Fig. 4E) in cortical neurons. Incubation of neurons with Aβ (5 μM; 2 hours) increased ROS levels to 124 ± 7.8 % and reduced the mitochondrial potential to 75 ± 3% with respect to control (100%, non-treated cells). Both parameters were partially restored by NMDA receptor antagonists AP5 (102 ± 11 % and 90 ± 2 %, respectively) and MK801 (104 ± 3.4 % and 85 ± 3 %, respectively) and by AMPA receptor antagonist CNQX (106 ± 6.3 % and 86 ± 3 %, respectively; Fig. 4D and E).

Taken together, these results demonstrate that both NMDA and AMPA receptors mediate Aβ-induced mitochondrial dysfunction.

Blockade of NMDA and AMPA receptors protect from Aβ-induced toxicity in entorhinal cortex-hippocampus organotypic slices

We next examined if Aβ oligomers induce Ca^{2+} and cell death in a more integral preparation. To that end, we used organotypic cultures from entorhinal cortex and hippocampus, two regions that are profoundly affected early in Alzheimer´s disease and cause cardinal symptoms of short-term memory loss (De Lacoste and White, 1993).

Slices for organotypic cultures were prepared as to preserve major connections between entorhinal cortex and hippocampus (Kluge et al., 1998). Thus, neurofilament immunofluorescence with antibodies to NFL revealed axons entering the hippocampus from the entorhinal cortex and layer preservation of these structures (Fig. 5A, B and C). In addition, antibodies to choline acetyltransferase show cholinergic fibers and terminals in entorhinal cortex (Fig. 5 D).

To monitor the effects of Aβ in these cultures, we measured Ca^{2+} levels after application of Aβ to neurons from CA1 in organotypic slices. We found that oligomeric Aβ (20 μM) increased $[Ca^{2+}]_i$ in those cells, and that this response was greatly reduced by MK801 (50 μM, n=9) and CNQX (30 μM, n=43) treatment (Fig 5E). Aβ-induced $[Ca^{2+}]_i$ decreased further by blocking simultaneously NMDA and AMPA receptors with MK801+CNQX (n=50; Fig 5E). In turn, Ca^{2+} responses elicited by kainate (1 mM) served as a positive control to evaluate the integrity of the culture (Fig. 5E).

Finally, to test Aβ toxicity we chronically incubated cultures with low concentrations of Aβ oligomers (100 nM) for 4 days, and used PI fluorescence to measure cell death. PI uptake in slices exposed to Aβ showed 4.1 ± 1.0 and 3.8 ± 0.9 fold over the control slices (n=7) in hippocampus and entorhinal cortex respectively (Fig. 5F-G). Instead, co-incubation of GluR antagonists MK801 (10 μM) or CNQX (30 μM) with Aβ oligomers reduced PI uptake to 0.9 ± 0.2 and 1.3 ± 0.4 fold in

hippocampus and to 1.2 ± 0.4 and 0.73 ± .01 fold in entorhinal cortex as compared to control slices treated with MK801 or CNQX alone (Fig 5F-G).

Overall, these results indicate that Aβ induces cytosolic Ca^{2+} overload and cell death in organotypic cultures, and that antagonists of NMDA and AMPA receptors prevent both effects.

Discussion

The results reported here show that Aβ oligomers dysregulate intracellular Ca^{2+} homeostasis through activation of both NMDA and AMPA receptors in neurons in vitro and in enthorinal cortex-hippocampus organotypic cultures. Ca^{2+} influx by GluRs produces mitochondrial dysfunction as indicated by mitochondrial Ca^{2+} overload, oxidative stress and mitochondrial membrane depolarization, which ultimately leads to apoptotic cell death. In turn, NMDA and AMPA receptor antagonists protect neurons against Aβ-mediated toxicity by attenuating Ca^{2+} influx and associated signaling cascades.

Several mechanisms may account for the Ca^{2+} mobilizing actions of Aβ oligomers in the experimental paradigm used in the current study. These include a direct interaction with membrane structure; insertion into the membrane to form a cation-conducting pore; activation of cell surface receptors coupled to Ca^{2+} influx; and oxidative stress leading to dysregulation of mitochondrial homeostasis (Wang et al., 2000, Arispe et al., 2007, De Felice et al., 2007, Sanz-Blasco et al., 2008). Our data show that increases in $[Ca^{2+}]_i$ induced by Aβ are mainly due to Ca^{2+} entry through the plasma membrane rather than release from intracellular Ca^{2+} stores as they were entirely prevented by removal of extracellular Ca^{2+}. In addition, we provide evidence that activation of NMDA and AMPA receptors by oligomeric Aβ is the primary and most important component related to Ca^{2+} dysregulation, mitochondrial alteration and cell death in neurons. Thus, Aβ oligomers applied alone or in conjunction with cyclothiazide, trigger inward currents that are abolished by NMDA or AMPA receptor antagonists, respectively. The amplitude of those currents is not affected by TTX or by removal of extracellular Ca^{2+} from bath solution, indicating that endogenous glutamate

release by Aβ oligomers does not contribute to receptor activation. Ca^{2+} imaging results confirmed that Aβ oligomers activate NMDA and AMPA receptors since the induced increase in $[Ca^{2+}]_i$ is greatly reduced by MK801, AP5 and memantine, and also by CNQX.

Consistent with the findings reported here, it has been suggested that Aβ interacts directly with NMDA and AMPA receptors and modulates channel properties. Thus, treatment of Aβ oligomers potentiate NMDA-evoked firing and induce a rapid and transient increase in intracellular Ca^{2+} levels that is blocked by memantine in mature hippocampal neurons (Szegedi et al., 2005; De Felice et al., 2007). In turn, Aβ oligomers have also been shown to induce Ca^{2+} influx, calpain activation and dynamin-1 degradation mediated by NMDA receptor activation (Kelly and Ferreira, 2006). In contrast, the reported effects of Aβ on AMPA receptors are apparently contradictory depending on the aggregation state of the peptide itself or the cell type studied. Thus, the fibrillar form of Aβ can robustly activate Ca^{2+} permeable AMPA receptor in neuronal cell lines (Blanchard et al., 2004) while it attenuates AMPA-evoked neuronal firing in CA1 neurons (Szegedi et al., 2005) and currents mediated by recombinant AMPA receptors expressed in Xenopus oocytes (Tozaki et al., 2002). The present study, however, indicates unambiguously that Aβ oligomers activate both NMDA and AMPA receptors which leads to Ca^{2+} dysregulation.

Our findings show that massive entry of Ca^{2+} into the cytosol induced by Aβ oligomers caused mitochondrial Ca^{2+} overload. This effect was absent in cells incubated previously with NMDA receptor antagonists indicating that Aβ oligomers promote a large and sustained entry of Ca^{2+} through NMDA receptors which is sufficient to activate the mitochondrial Ca^{2+} uniporter. Mitochondrial Ca^{2+} overload during

excitotoxicity favors ROS production and mitochondrial membrane depolarization (revised in Atlante et al., 2001). Consistent with that, we also observed these two deleterious features after treatment with Aβ oligomers, and that both are prevented by NMDA and AMPA receptor antagonists.

Increased levels of free radicals and Ca^{2+} overload in mitochondria lead to the release into the cytoplasm of proapoptotic factors (Nicholls and Budd, 2000). Cytochrome c and the apoptosis-inducing factor are released from mitochondria to the cytosol and the nucleus, in which they induce activation of caspase-3 and PARP-1. In the current study, the fact that caspase-3 and PARP1 inhibition rescue neurons from Aβ oligomeric toxicity strongly suggests that Aβ kills neurons by apoptosis involving both caspase-dependent and independent pathways.

Finally, we observed that Aβ oligomers induce neurotoxicity in cortical cell culture, and in entorhinal cortex and hippocampal organotypic slices. That toxicity was attenuated by NMDA and AMPA receptor antagonists in the two preparations assayed. Of all vulnerable regions, pyramidal neurons of the neocortex, entorhinal cortex and hippocampus are affected early in the disease process (Butterfield and Pocernich, 2003; Cacabelos et al.1999, Francis, 2003). It also has been proposed that a sustained increase in extracellular glutamate levels and activation of the NMDA receptor is associated with the cognitive deficits and loss of neurons observed in AD brains (Mattson and Chan, 2003; Hynd et al., 2004). Our current results indicate that excessive NMDA and AMPA receptor activity in AD may be a consequence of Aβ interaction with those receptors in crucial neuronal circuitries early in the disease.

In summary, our data point to dysregulation of intracellular Ca^{2+} homeostasis underlies Aβ bloomer toxicity. Aβ peptides bind to NMDA and AMPA receptors disturbing the cytosolic and mitochondrial Ca^{2+} levels. Mitochondrial Ca^{2+} overload

triggers mitochondrial dysregulation that induces apoptosis by caspase-dependent and independent pathways. Drugs that modulate these events can prevent from Aβ damage to neurons in Alzheimer´s disease.

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Legends

Figure 1. Aβ oligomers activate NMDA receptors and induce Ca^{2+} influx into neurons. ***A,*** Aβ (1 μM) evokes inward, non-desensitizing currents when applied together with glycine in Mg^{2+}-free medium. Aβ-activated currents are blocked by AP5 (100 μM), a selective NMDA receptor antagonist. TTX (1 μM) and calcium-free extracellular bath solution do not attenuate Aβ-induced inward currents. ***B,*** Aβ (5 μM) induces a rapid increase in $[Ca^{2+}]_i$, an effect that is blocked by NMDA receptor antagonists, MK801, AP5 and memantine, or by removal of $Ca^{2+}$from the perfusate. Voltage-gated Ca^{2+} channel inhibitors nifedipine and verapamil and CNQX do not diminish Aβ response.

Figure 2. Aβ oligomers activate AMPA receptors and increase $[Ca^{2+}]_i$ in neurons. ***A,*** Aβ (1 μM) together with CTZ (100 μM) evokes inward, non-desensitizing currents in the presence of Mg^{2+}. Currents are blocked by the AMPA/kainate receptor antagonist CNQX (30 μM). ***B,*** Aβ in conjunction with CTZ induces $[Ca^{2+}]_i$ increase, that is partially blocked by CNQX and AP5, and further reduced by both antagonists applied together.

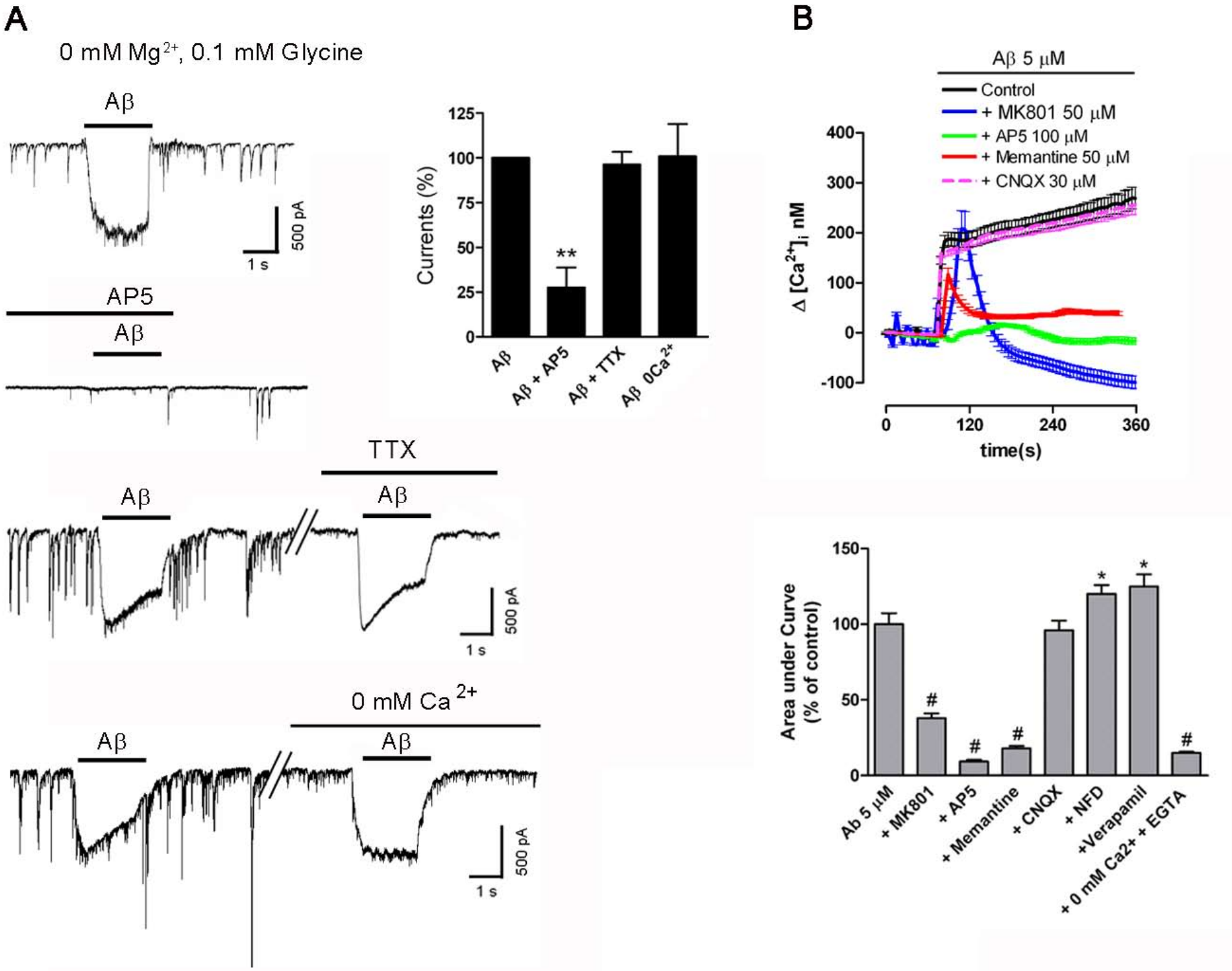
Figure 3. Aβ oligomers trigger neuronal apoptotic death by activating NMDA and AMPA/kainate receptors. ***A,*** Dose-response toxicity of Aβ oligomers in cortical neurons in culture as measured 24 h later with the LDH viability assay. ***B,*** Toxicity is prevented by co-application of Aβ oligomers (5 μM) with NMDA receptor antagonists AP5 (100 μM), MK801 (50 μM) and memantine (50 μM), and with AMPA/kainate receptor antagonist CNQX (30 μM). ***C,*** Aβ induced-toxicity was reduced by the broad-

spectrum caspase-inhibitor ZVAD (50 μM), by caspase-3 inhibitor DEVD (100 μM) and by PARP-1 inhibitor DPQ (30 μM). (* $p< 0.05$, ** $p < 0.01$ for each comparison between Aβ alone and the co-applied with GluR antagonist or the corresponding inhibitor).

Figure 4. Blockade of glutamate receptors attenuates Aβ-induced mitochondrial alterations. Neurons, loaded with Rhod-2 AM, were exposed to ***A*** FCCP (25 μM) used as an internal control to release Ca^{2+} from mitochondria, or to ***B*** Aβ alone (5 μM) or together with NMDA and AMPA receptor antagonists. ***B*** and ***C,*** $[Ca^{2+}]_{mit}$ induced by Aβ is reduced by NMDA receptor antagonists. Recordings in B illustrate average ± SEM responses of 74 cells from at least 5 experiments, and histogram represents average ± SEM of area under Ca^{2+} curve for each condition. ***D*** and ***E***, Cultures were first exposed to Aβ alone or in conjunction with GluR antagonists for 2 hours, and cells were immediately loaded with the corresponding dyes to monitor ROS generation and mitochondrial depolarization by fluorimetry. NMDA and AMPA/kainate receptor antagonists prevent the increase in radical oxygen species and mitochondrial depolarization produced by Aβ. * $p < 0.05$, ** $p < 0.01$ for each antagonist versus Aβ.

Figure 5. MK801 and CNQX reduce Aβ-induced $[Ca^{2+}]_i$ rises and abolish cell death in entorhinal cortex-hippocampus organotypic cultures. Cultures were fixed at 7 DIV and characterized by immunofluorescence with antibodies to neurofilaments (NFL) and cholinergic terminals (ChAT). ***A, B,*** Hippocampus and entorhinal cortex showed profuse staining with NFL antibody. ***C,*** Square shows the magnification in A of neuronal fibers from entorhinal cortex entering in CA1. ***D,*** Cholinergic fibers revealed with ChAT in the entorhinal cortex (ECx) at high magnification. ***E,*** Aβ (20 μM)

induces a rapid increase in $[Ca^{2+}]_i$ in hippocampus, an effect that is blocked by NMDA receptor antagonists MK801 (50 μM; n=9) and by AMPA receptor antagonist CNQX (30 μM; n=43) and further reduced by both antagonists applied together (n=50). Kainate (Kai) 1 mM also increases $[Ca^{2+}]_i$ in the same selected neurons. ***F, G*** Histogram and representative fields showing Aβ (100 nM, 4 days) toxicity in cultures treated after 7 DIV and protection when oligomers are applied in conjunction with MK801 (10 μM) or CNQX (30 μM). Scale bar in G represent 500 μm. ***F,*** Bars (gray value/area) represent neuronal cell death occurs in Aβ-treated hippocampus and entorhinal cortex. MK801 and CNQX mainly blocked the cell damage in both areas. Bars in F represent the average ± SEM from 5 experiments. # < 0.05 for comparison Aβ vs control treatments. * $p < 0.05$ for comparison of Aβ vs antagonist treatment .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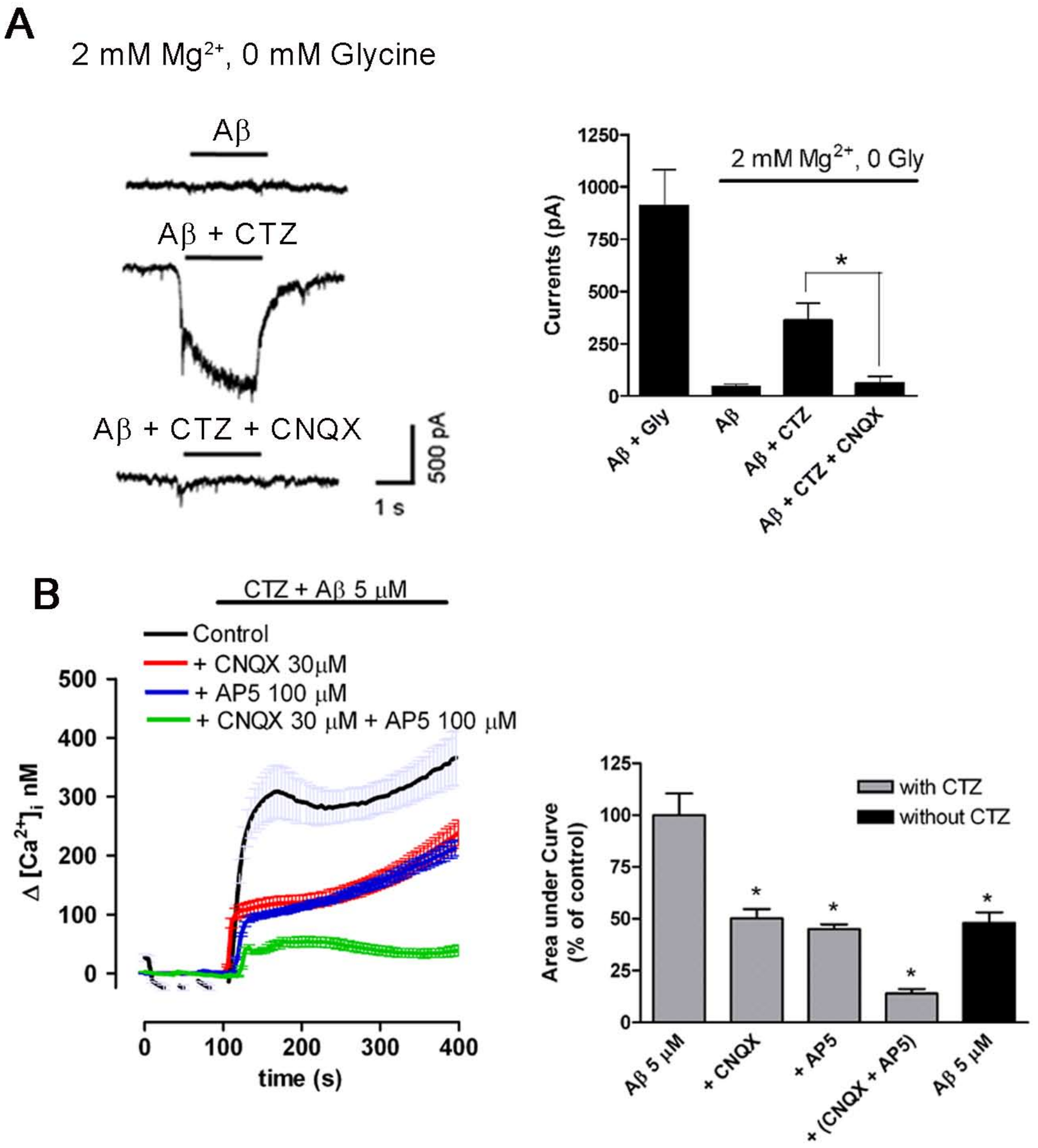
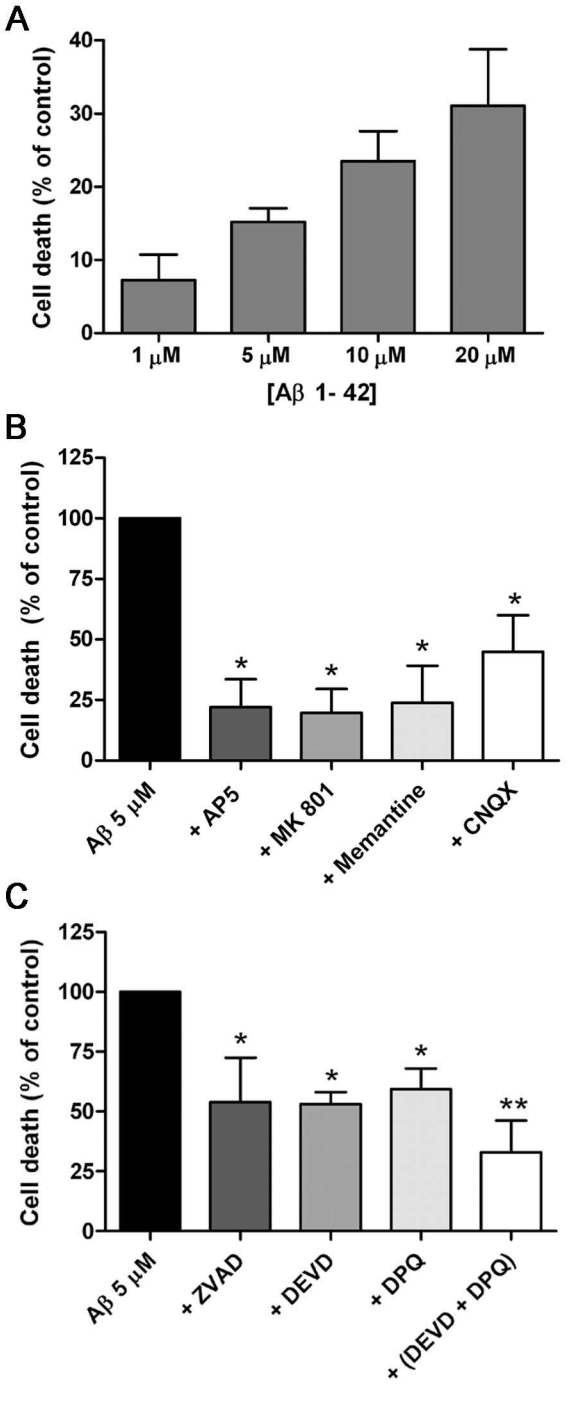
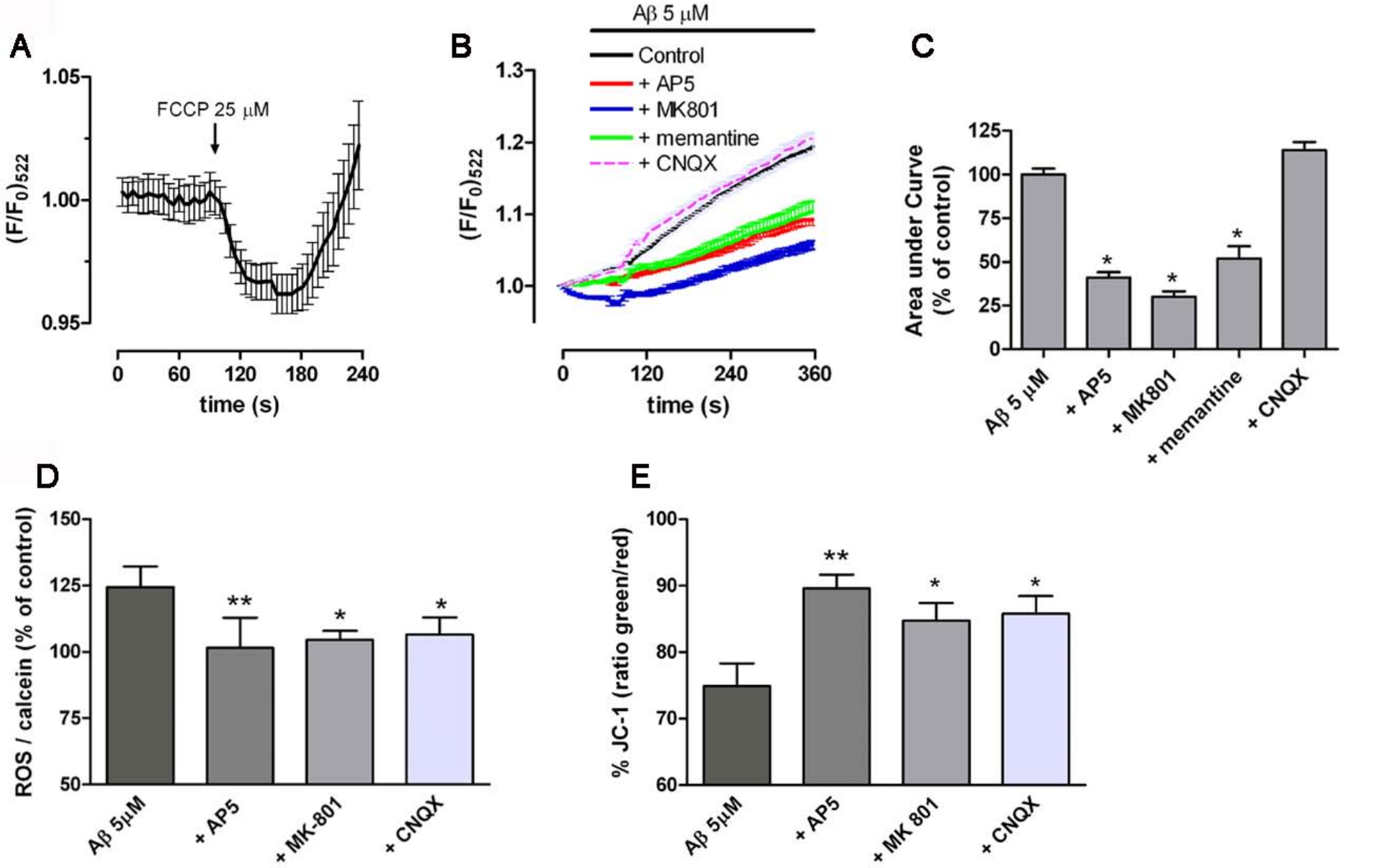
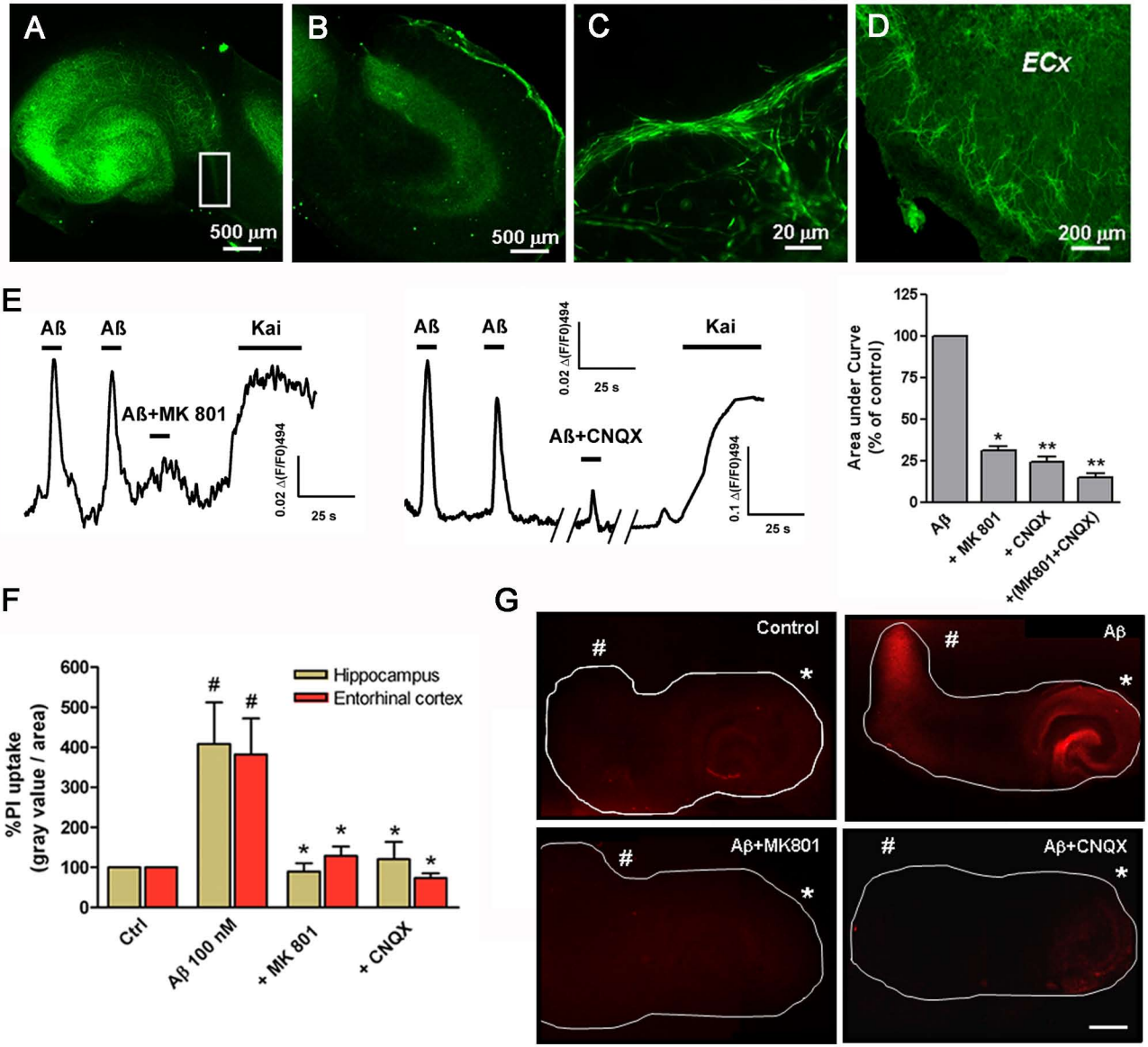
0 mM Mg2+, 0.1 mM Glycine
Aβ
500 pA
1 s
AP5
Aβ
TTX
Aβ
Aβ
500 pA
1 s
0 mM Ca 2+
Aβ
Aβ
500 pA
1 s
Currents (%)
125
100
75
50
25
0
**
Aβ
Aβ + AP5
Aβ + TTX
Aβ 0Ca2+
B
Aβ 5 μM
Control
+ MK801 50 μM
+ AP5 100 μM
+ Memantine 50 μM
+ CNQX 30 μM
Δ [Ca2+]i nM
400
300
200
100
0
-100
0
120
240
360
time(s)
Area under Curve
(% of control)
150
100
50
0
#
#
#
*
*
Ab 5 μM
+ MK801
+ AP5
+ Memantine
+ CNQX
+ NFD
+Verapamil
+ 0 mM Ca2+ + EGTA


A
2 mM Mg2+, 0 mM Glycine
Aβ
Aβ + CTZ
Aβ + CTZ + CNQX
500 pA
1 s
2 mM Mg2+, 0 Gly
Currents (pA)
1250
1000
750
500
250
0
*
Aβ + Gly
Aβ
Aβ + CTZ
Aβ + CTZ + CNQX
B
CTZ + Aβ 5 μM
Control
+ CNQX 30μM
+ AP5 100 μM
+ CNQX 30 μM + AP5 100 μM
Δ [Ca2+]i nM
500
400
300
200
100
0
0
100
200
300
400
time (s)
Area under Curve
(% of control)
125
100
75
50
25
0
with CTZ
without CTZ
*
Aβ 5 μM
+ CNQX
+ AP5
+ (CNQX + AP5)
Aβ 5 μM


A
Cell death (% of control)
40
30
20
10
0
1 μM
5 μM
10 μM
20 μM
[Aβ 1- 42]
B
Cell death (% of control)
125
100
75
50
25
0
*
*
*
*
Aβ 5 μM
+ AP5
+ MK 801
+ Memantine
+ CNQX
C
Cell death (% of control)
125
100
75
50
25
0
*
*
*
**
Aβ 5 μM
+ ZVAD
+ DEVD
+ DPQ
+ (DEVD + DPQ)


A
FCCP 25 μM
(F/F0)522
time (s)
B
Aβ 5 μM
Control
+ AP5
+ MK801
+ memantine
+ CNQX
(F/F0)522
time (s)
C
Area under Curve (% of control)
Aβ 5 μM
+ AP5
+ MK801
+ memantine
+ CNQX
D
ROS / calcein (% of control)
Aβ 5μM
+ AP5
+ MK-801
+ CNQX
E
% JC-1 (ratio green/red)
Aβ 5μM
+ AP5
+ MK 801
+ CNQX


A
500 µm
B
500 µm
C
20 µm
D
ECx
200 µm
E
Aß
Aß
Kai
Aß+MK 801
0.02 Δ(F/F0)494
25 s
Aß
Aß
Kai
Aß+CNQX
0.02 Δ(F/F0)494
25 s
0.1 Δ(F/F0)494
25 s
Area under Curve (% of control)
125
100
75
50
25
0
Aß
+ MK 801
+ CNQX
+(MK801+CNQX)
F
%PI uptake (gray value / area)
600
500
400
300
200
100
0
Hippocampus
Entorhinal cortex
Ctrl
Aß 100 nM
+ MK 801
+ CNQX
G
Control
Aβ
Aβ+MK801
Aβ+CNQX