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β-Carbolines induce apoptosis in cultured cerebellar granule neurons via the mitochondrial pathway

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Abbreviations

βC: β-carboline; βCCB: β-carboline-3-carboxylic acid ester; $\Delta\Psi_m$: mitochondrial membrane potential ; AIF: apoptosis-inducing factor; BA: bongkrekic acid; CGN: cerebellar granule neuron; CyA: cyclosporin A; IMM: inner mitochondrial membrane; MTT: 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazam bromide ; OMM: outer mitochondrial membrane; PTP: permeability transition pore; VDAC: voltage-dependent anion channel.

Running title

β-Carbolines induce intrinsic apoptotic pathway

Abstract

N-butyl-β-carboline-3-carboxylate (βCCB) is, together with 2-methyl-norharmanium and 2,9-dimethylnorharmanium ions, an endogenously occurring β-carboline. Due to their structural similarities with the synthetic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), harman and norharman compounds have been proposed to be involved in the pathogenesis of Parkinson's disease. While also structurally related, βCCB has received much less interest in that respect although we had previously demonstrated that βCCB induces the apoptotic cell death of cultured cerebellar granule neurons (CGNs). Herein, we have investigated the molecular events leading to CGN apoptosis upon βCCB treatment. We first demonstrated that βCCB-induced apoptosis occurs in neurons only, most likely as a consequence of a specific neuronal uptake as shown using binding/uptake experiments. Then we observed that, in βCCB-treated CGNs, caspases 9, 3 and 8 were successively activated, suggesting an activation of the mitochondrial pathway. Consistently, βCCB also induced the release from the mitochondrial intermembrane space of two pro-apoptotic factors, *i.e.* cytochrome c and apotptosis inducing factor (AIF). Interestingly, no mitochondrial membrane depolarisation was associated with this release, suggesting a mitochondrial permeability transition pore-independent mechanism. The absence of any neuroprotective effect provided by two mPTP inhibitors, *i.e.* cyclosporine A and bongkrekic acid, further supported this hypothesis. Together, these results show that βCCB is specifically taken up by neuronal cells where it triggers a specific permeabilization of the outer mitochondrial membrane and a subsequent apoptotic cell death.

Keywords

Apoptosis, mitochondrion, caspases, β-carbolines, cerebellar granule neurons

Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative disorders that affects more than one percent of the population over the age of 60. PD is mainly a sporadic disease although some genetic and toxic forms have been described. The prototypic toxic form of PD is caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a meperidine analogue. In the brain, MPTP is oxidized into MPP+ by monoamine oxidase B which is abundantly expressed by glial cells. MPP+ is subsequently and specifically taken up by dopaminergic neurons where it induces a dysfunction of the complex I of the respiratory chain. Because a deficit in complex I function has also been described in PD patients, MPP+ toxicity is widely used as a model of PD (Eberhardt et al., 2003).

Some environmental pyro-indoles (carbolines) are structurally close to MPTP and MPP^+ when they are N-methylated. Among carbolines, β-carbolines (βCs), including harman and norharman compounds, have received most attention in the field of PD research(Collins et al., 2002). Some studies indeed suggest that βCs could, as MTPP, be metabolized *in vivo* and, hence, lead to the formation of a toxin similar to MPP^+. Moreover, βCs are toxic for dopaminergic neurons and reproduce PD-like symptoms when injected into rodents(Matsubara et al., 1998). In humans, the concentration of two βCs, *i.e.* 2,9-dimethylnorharmanium and 2-monomethylated norharmanium, seems to be increased in the CSF of patients with idiopathic Parkinson's disease compared to control subjects (Matsubara et al., 1995). This led to the emergence of a theory according to which exposure to some environmental toxins, including βCs, would contribute to the pathogenesis of PD.

βCs structurally related to harman and norharman compounds also deserve other function in the nervous system. For example, some are well characterized negative modulators at

inhibitory ionotropic receptors, such as the type A receptor for γ-aminobutyric acid receptor ($GABA_AR$)(Rigo et al., 1994) or the strychnine-sensitive glycine receptor(Rigo et al., 2002). Among these, N-butyl-β-carboline-3-carboxylate ester (βCCB), has also been shown to occur in mammalian tissues(Braestrup et al., 1980), including the brain(Pena et al., 1986; Pena et al., 1988). Relation between βCCB and neuronal degeneration has never been investigated *in vivo,* but we have previously shown that, *in vitro,* βCCB was able to induce neuronal apoptosis(Malgrange et al., 1996).

Since βCCB is a potentially endogenous compound structurally related to MPP+ and is able to induce an apoptotic death in cultured cerebellar granule neurons (CGNs), we decided to decipher the molecular pathway leading to apoptosis in this model. CGNs are among the most homogenous neuronal cultures that can be obtained from the rat central nervous system since they consist of an homogenous neuronal subtype, *i.e.* cerebellar granule neurons, and since their overall glial content was found not to exceed 5 %(Lefebvre et al., 1987). CGNs have not only been widely used as a model to study neuronal apoptosis induced by various toxins, but were also found to adhere to all the criteria established for studying PD-related toxins, including the MPP+(Gonzalez-Polo et al., 2001; Marini et al., 1989; Kalivendi et al., 2003). They were also successfully used to study apoptotic mitochondrial events, including mitochondrial membrane potential changes (Gonzalez-Polo et al., 2003).

From a molecular point of view, apoptosis is a complex type of cell death which can be triggered by numerous stimuli acting at as many death sensors. It is usual to distinguish the extrinsic pathway from the intrinsic pathway. The former is initiated through the activation of membrane death receptors by specific ligands(Ashkenazi et al., 1999), which in turn triggers the activation of caspase 8(Earnshaw et al., 1999). Activation of the intrinsic pathway, on the other hand, requires an increase in the mitochondrial membrane permeability that leads to the

release of mitochondrial pro-apoptotic factors, including cytochrome c and apoptosis inducing factor (AIF)(van Loo et al., 2002). Once in the cytosol, cytochrome c binds to the scaffolding protein apaf-1 to form the apoptosome which activates caspase 9(Adams et al., 2002). Regarding AIF, it translocates to the nucleus where it contributes to apoptotic DNA fragmentation(Cande et al., 2002). Finally, both initiator caspases, caspase 8 and caspase 9, activate the effector caspase 3, which eventually initiates the machinery leading to apoptotic cell death(Fischer et al., 2003).

In this work, we have investigated the molecular events leading to neuronal apoptosis upon βCCB treatment. We first demonstrated that βCCB-induced apoptosis occurs in neurons only, most likely as a consequence of a specific neuronal uptake. Then we observed that βCCB-induced neuronal death was dependent upon caspase 9 and caspase 3 activation. This pattern of caspase activation suggested a mitochondrial implication. This was confirmed by the finding that two mitochondrial pro-apoptotic factors, *i.e.* cytochrome c and AIF, were released into the cytosol. Interestingly, the mitochondrial release of these factors was not associated with any $\Delta\psi_m$ loss suggesting a selective OMM permeabilization.

Material and methods

Chemicals

Butyl β-carboline-3-carboxylate (βCCB) was purchased from Tocris (UK). Pancaspase inhibitor Boc-D-FMK, caspase 9 inhibitor Z-Leu-Glu(Ome)-His-Asp(Ome)-Fluoromethylketone, caspase 3 inhibitor Z-Asp-Glu(Ome)-Val-Asp(Ome)-Fluoromethylketone and caspase 8 inhibitor Z-Ile-Glu(Ome)-Thr-Asp(Ome)-Fluoromethylketone were all from Calbiochem, San Diego, CA, USA. Bongkrekic and Cyclosporine A were also provided by Calbiochem, San Diego, CA, USA. The mitochondrial membrane potential sensitive dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanide iodide) and the mitochondrial probe MitoTracker red 580 were from Molecular Probes, Leiden, Netherlands. The MTT 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazam bromide was from Sigma-Aldrich.

Cell cultures

Neuronal cultures

Cerebellar granule neurons (CGNs) were obtained from 7-day-old rat pups as previously described (Malgrange et al., 1996; Rigo et al., 1994). Briefly, cerebella were removed and freed of meninges. They were then minced into small fragments and incubated at 37°C for 25 minutes in 0,25% trypsin and 0,01% DNAase (in a cation-free solution). Fragments were then washed with minimum essential medium (GIBCO, Ghent, Belgium) supplemented with glucose (final concentration 6 g/l), insulin (Sigma; 5 µg/mL), pyruvate (GIBCO, Ghent, Belgium; 1 mM) and Horse serum (HS, GIBCO, 10 % v/v). The potassium concentration was increased to 25 mM while the sodium concentration was decreased in equimolar amount

(MEM-25HS). The dissociation was achieved mechanically by up and down aspirations in a 5 ml plastic pipette. The resulting cell suspension was then filtered on a 15 µm nylon sieve. Cells were then counted and diluted to a final concentration of $2.5x10^6$ cells per ml. The resulting cell suspension was transferred to a T75 flask (falcon®, Bedford, MA, USA) and incubated for 2 hours at 37°C. After two hours, most glial cells adhere to the bottom while neurons are resuspended by 20 minutes shaking at 100 RPM. The cell suspension was then plated on poly-ornithine- (0.1 mg/ml) coated substrates. The cells were cultured for 24 hours before experimental procedures were performed. Previous immunocytochemical studies have shown that such cultures contain 95 % of neurons (Lefebvre et al., 1987).

Astrocyte cultures

Astrocytes were cultured from newborn rat cerebral cortex (Booher et al., 1972). Cerebral hemispheres were dissected, carefully freed of meninges and dissociated by sieving through a nylon mesh (pore size = 225 µm). Cells were collected in MEM supplemented with 10% (v/v) FCS, bovine insulin (5 µg/mL) and glucose (6 g/l, final concentration) and filtered through a second nylon mesh (pore size = 25 µm). The cell suspension obtained from one animal was seeded into one plastic Petri dish (100 mm diameter). More than 93% of the cells in these cultures were immunopositive for glial fibrillary acidic protein (Lefebvre et al., 1987). After one week of culturing, cells were subcultivated in 96-wells microplate for toxicity assays or in 24-wells plates for binding/uptake assays.

Determination of Cell survival

For cell survival determination, 50 µl of the cell suspension were plated in 96-well microplates (NUNC, Roskilde, Denmark). After 24 hours, the medium of CGN or astrocyte cultures was removed and replaced by MEM containing low potassium (5.4 mM) and

supplemented with glucose (final concentration 6 g/l), insulin (5 µg/ml) and pyruvate (1 mM) (MEM-5). This medium was supplemented with the drugs to be tested or with equal amount of vehicles. Vehicles were ethanol for βCCB (stock solution at 10^{-2} M) or DMSO for caspases inhibitors (stock 20 mM). After a 24-hour exposure to drugs, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazam bromide (MTT) was added to the medium at a final concentration of 0.15 mg/ml. This compound is converted into an insoluble blue formazam product by mitochondria of living cells but not of dying cells or debris. After two hours of incubation, the medium was carefully removed and the blue precipitate dissolved in isopropanol by up and down aspiration using a multi-channel pipette. The light absorbance which is proportional to the cell survival was read using a Biotek EL-309 ELISA reader (test wavelength 540 nm, reference wavelength 650 nm).

[^{3}H]βCCB binding/uptake assay

Cell cultures (in 24-well plates) were rinsed with PBS and stored for 30 min at 4°C. All experimental points were done in triplicate. [^{3}H]βCCB was custom-labelled by DuPont-NEN. The cells were incubated in the presence of [^{3}H]βCCB in PBS for 60 min at 4°C (this time corresponded to 4 times the half-life of association kinetics of [^{3}H]βCCB determined in a separate experiment; data not shown). Incubation was terminated by three rinses with PBS added with 10 µM unlabelled βCCB. Cells were then digested in 0.1 N NaOH (1 ml per well) for 10 min at 37°C. The digestion product was neutralized with 10 µl of 12 N HCl and placed in scintillation flasks. Seven millilitres scintillation liquid were added and radioactivity in the flasks was determined by conventional liquid scintillation counting using a Beckman LS3801 counter. For dissociation kinetics determination, cells were incubated in PBS for various periods of time before being digested. Non specific binding/uptake was determined using

10 μM unlabelled βCCB.

Western blotting

CGN were cultured as described above in 35-mm plastic Petri dishes (NUNC) at 2.5 X 10^6 cells per dish. After various times of treatment with drugs (see above), cells were lysed in a buffer containing 25 mM Hepes, 150 mM NaCl, 0.5% Triton, 10% Glycerol, 1 mM DTT, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 1 mM NaF and complete® protease inhibitor cocktail (1 tablet for 50 ml; Roche, Brussels, Belgium). The protein content of each lysate was determined by using the Bradford method as previously described (Bradford 1976). Protein lysates were then mixed with an equal volume of gel loading buffer (20% glycerol, 4%SDS, 100 mM Tris, 5% β-mercaptoethanol and bromophenol blue) before being boiled for 3 minutes. After boiling, 30 μg of protein were subjected to SDS-PAGE gel electrophoresis. Proteins were then transferred on a PVDF membrane (Amersham, Roosendaal, the Netherlands) by semi-dry electroblotting in transfer buffer (192 mM glycine, 25 mM Tris and 20% methanol). Blots were then blocked overnight at 4°C in 10% non-fat dry milk diluted in TBS supplemented with 0.05% Tween 20 (Bio-Rad, Nazareth, Belgium) (TTBS). The following primary antibodies were incubated for 2 hours at room temperature in TTBS supplemented with 5% non-fat dry milk as follows: rabbit polyclonal anti-caspase 3 (sc-7148, Santa Cruz Biotechnology, 1:5000), rabbit polyclonal anti-caspase 9 (sc-8355, Santa Cruz Biotechnology, 1:5000), rabbit polyclonal anti-caspase 8 (Catalog N° 559932, BD PharMingen, 1:5000) and mouse monoclonal anti-β-actin (Sigma-Aldrich, Belgium, 1:5000). Peroxidase-conjugated secondary antibodies were incubated for 1 hour at room temperature as follows: monoclonal anti-rabbit (clone RG-16, Sigma Aldrich, 1:5000) and goat anti-mouse IgG (Product N° A2304, Sigma Aldrich, 1:5000). Blots were then washed extensively and

developed using enhanced chemoluminescence (Pierce, Aalst, Belgium). The band intensity was analysed by using the ImageMaster 1D software v3.01 (Amersham, Roosendaal, The Netherlands).

Caspase activation assay

CGN cultures were performed on 10-mm diameter glass coverlips placed in 4-wells tissue culture dishes (NUNC). After various times of treatment with drugs (see above), CGN cultures were subjected to a caspase activity detection kit (Calbiochem, San Diego, CA, USA). This procedure consisted of treating the cultures for 1 hour at 37°C with appropriate FITC-conjugated caspase inhibitors applied at a final concentration of 5 μM (1:300 dilution from stock solution) in MEM-5, *i.e.* FAM-LETD-FMK (caspase 8), FAM-LEHD-FMK (caspase 9) and FAM-DEVD-FMK (caspase 3). Cultures were then rinsed three times with the wash buffer of the caspase activity detection kit diluted at 1:10 (v/v) in tridistilled water. Cells were fixed for 10 minutes in 4% paraformaldehyde, rinsed three times with PBS and counterstained with the nuclear dye Ethidium homodimer (Live/Dead® Viability/Cytotoxicity kit, Molecular Probes, Leiden, Netherlands) diluted at 1:5000 (v/v) in PBS. The cultures were finally washed three times with PBS and mounted in Fluoroprep (Biomerieux, France).

Immunocytochemistry for the detection of cytochrome c and AIF

Cultures were made as for the caspase activation assay (see above). After treatment with drugs, cells were fixed for 10 minutes in 4% paraformaldehyde. They werewashed three times with PBS and permeabilized for 5 minutes in 1% (v/v in PBS) Triton X-100. Non-specific binding was blocked by a 45–minute treatment at room temperature in a PBS solution containing non-fat dry milk (15 mg/ml). Cells were subsequently incubated for 1.5 hours at room temperature with primary antibodies diluted in non-fat dry milk-containing PBS. These

were purified mouse monoclonal IgG anti-cytochrome c (dilution 1:500, Pharmingen®, clone 6H2.B4) and rabbit polyclonal anti-AIF (dilution 1:100, Chemicon®). After three washes in PBS, cultures were incubated for 45 minutes at room temperature with the following secondary antibodies: FITC-conjugated donkey anti-mouse IgG and FITC-conjugated anti-rabbit IgG (both at 1:500 dilution in non-fat dry milk-containing PBS; antibodies were from Jackson ImmunoResearch Laboratories). Cells were finally washed three times in PBS and counterstained either with the nuclear dye ethidium homodimer as described above or with Mitotracker red 580 (Molecular Probes, Leiden, Netherlands) diluted at 100 nM in PBS for 10 minutes before being mounted in Fluoroprep (Biomerieux, France).

Confocal microscopy

Cells subjected to the caspace detection kit or to immunocytochemistry were imaged using a MRC 1024 laser scanning confocal microscope (Bio-Rad, Hertz, UK) equipped with a mixed argon-krypton laser. The Laser Sharp software was used for data acquisition. The voltage of each photomultiplier tube, the iris and the black level were kept constant during one given experimental procedure. All pictures represent single optical plane section of 0.5 µM thickness.

Mitochondrial membrane potential assessment using JC-1

To assess the mitochondrial membrane potential, two JC-1 assays were performed. The first one used confocal microscopy. For that purpose, cell were cultured in LabTek II 8-wells glass chamber slides with covers (Nagle Nunc international, Naperville, IL, USA) and treated as described above. After various times of exposure to drugs, the medium was removed and cells were loaded with 5 µg/ml JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanide iodide, , Molecular Probes) in MEM-5 for 30 minutes

at 37°C. This dye concentrates as aggregates in mitochondria that maintain a high membrane potential ($\Delta\Psi_m$). Conversely, in mitochondria presenting a membrane depolarisation, the dye accumulates as monomers. Upon excitation at 488 nm, aggregates have a red emission (~590 nm) while monomers have a green emission (~525 nm) (Dedov et al., 2001; Salvioli et al., 1997; Cossarizza et al., 2001). For each cell in a given microscopic field, red and green intensities of the cytoplasm were determined using the Laser Sharp software. A green/red ratio was then calculated which corresponds to the ratio of JC-1 monomeres/aggregates and, hence, is proportional to the mean $\Delta\Psi_m$ of the cell mitochondria. For each condition, a green/red ratio was calculated for at least 30 cells and subsequently normalised to the mean green/red ratio of corresponding controls.

The second JC-1 assay consisted of a measure that used an fluorescence-activated cell sorter (FACS). For that purpose, CGNs were cultured in 35-mm Petri dishes and treated as described above. After 12 hours in the experimental conditions, cells were incubated with 5 µg/ml JC-1 in PBS supplemented with glucose (6 g/l) for 30 minutes at 37°C. They were then rinsed three times with PBS and transferred on ice. Cells were scraped from the bottom of the dishes and re-suspended in PBS. Cytofluorometric analyse was performed within 10 minutes by using a FACSort instrument (Becton Dickinson). After excitation at 488 nm, emission in FL1 corresponds to JC-1 monomers and in FL2 to JC-1 agreggates. The results were analysed using the Cellquest program (Becton Dickinson).

Statistical analyses

For each type of experiments, results of at least two independent procedures were pooled and expresses as mean ± SEM except stated otherwise. Comparison between two mean values was performed using unpaired *t*-test. When more than two mean values were compared, a one-way

analysis of variance (ANOVA) was used with Dunnett's post-tests when significance was reached.

RESULTS

βCCB-induced death is neuron-specific

We have already reported that several β-carbolines induce the death of cultured cerebellar granule neurons (CGNs) (Malgrange et al., 1996). It was however not known if this effect was neuron-specific. **Figure 1** clearly show that, while incubating cultured CGNs with increasing βCCB concentrations resulted in a concentration-dependent loss of neuronal viability, the survival of cultured astrocytes was only poorly affected. Indeed, at 100 µM βCCB, 66 ± 10 % (n = 4) of astrocytes still survived, while 40 µM βCCB treatment of CGNs resulted in a complete neuronal loss ($P<0.05$ ANOVA followed by Dunnett's post-tests). The concentration that induced 50 % of CGN death (IC_{50}) was 28.4 ± 2.7 µM (n = 8).

βCCB can be accumulated intracellularly and this process is also neuron-specific

In our previous report (Malgrange et al., 1996), the molecular cascade leading to neuronal death was not investigated although some arguments suggested the involvement of an apoptotic pathway. Since initiation of an apoptotic cascade could be both membrane- and intracellularly (mainly mitochondrion-) triggered – corresponding to the extrinsic and intrinsic pathways, respectively –, we examined the ability of βCs to bind and/or to accumulate within cultured CGNs.

For that purpose, we first determined the binding/uptake characteristics of custom-labelled [^{3}H]βCCB in cultured CGNs. **Figure 2A** shows the binding/uptake isotherm and Scatchard transformation of [^{3}H]βCCB. Derived K_D (expressed in 1:100000 dilution from the stock

solution) and B_{max} values were of 1.5 (isotherm) / 2.6 (Scatchard) and 7997 (isotherm) / 10450 (Scatchard) dpm (corrected for proteins), respectively. Competition between unlabelled βCCB and [^{3}H]βCCB (1:20000 dilution from the stock solution) yielded a K_i of 65.6 nM if a single competition curve was fitted, but data were better described (F-test with $P<0.05$) by a two-site competition curve yielding K_i of 233.1 (62.5%) and 13.2 nM (**figure 2B**).

We proceeded to compare the dissociation/release kinetics of preloaded [^{3}H]βCCB from CGNs, astrocytes and 3T3 fibroblasts in culture (**figure 2C**). No specific binding/uptake could be measured in 3T3 fibroblasts. In astrocytes, the initial binding/uptake was 43.8% of the one measured in neurons and disappeared totally after a 30 min washout (fitting of the data yielded a half-life of 5.3 min and a plateau not significantly different from 0; $P>0.05$ two-way ANOVA). By contrast, in neurons, the radioactivity specifically associated with the cells decayed (half-life = 3.8 min) to a plateau that still represented 40.0% of the initial radioactivity, suggesting the existence of a very slow exchangeable compartment that trapped [^{3}H]βCCB. The level of this slowly exchangeable compartment could be modulated both negatively (by raising the binding/uptake temperature from 4 to 37°C; data not shown) and positively (by depolarizing the CGNs; see **figure 2D**). These data suggest that βCCB could accumulate within CGNs, but not in astrocytes, in a depolarization-dependent process and, hence, that its primary target might be intracellular.

βCCB activates caspases of the mitochondrial apoptotic pathway

To ascertain that βCCB-induced death of CGNs was apoptotic and to get some insight into the molecular cascade involved, we looked for caspase activation upon βCCB treatment.

Caspase activation in βCCB-treated CGNs was first assessed by using specific fluorometric

caspase activity assays since they allow a morphological identification of the cells where caspases are activated. When cultured CGNs were treated for 10 hours with 30 µM βCCB, a significant increase in caspase 9 activity was observed (12.8 ± 1.4% of cell presenting a caspase 9 activation compared to 2.3 ± 0.1% in controls; n = 3 experiments; $P<0.05$, unpaired t-test; **figures 3H**), while no change was noticed both for caspase 3 and caspase 8 activities ($P>0.05$; unpaired *t*-test; **figures 3G** and **3I**). After 12 hours of treatment, a significant increase in both caspase 3 and caspase 9 activities was observed in βCCB-exposed cultures compared to controls (**figures 3A** to **3D**). At that time, the percentage of neurons presenting a caspase 3 activation was 80.2 ± 4.3 (n = 3 experiments; $P<0.001$ ANOVA followed by Dunnett's post-tests) and the proportion of neurons displaying caspase 9 activation was 43.1 ± 5.2% ($P<0.001$ ANOVA followed by Dunnett's post-tests; **figures 3G** and **3H**). Conversely, no detectable caspase 8 activity was observed in βCCB-treated cultures even after 12 hours (**figures 3E** to **3I**).

Caspase activation in βCCB-induced CGN death was further confirmed by time-dependent western blot analysis of pro-caspases 9, 3 and 8 (**figure 3K**). The intensities of the pro-caspase signals were measured and expressed as percentage of the corresponding signals in control conditions to further ascertain the sequence of activation (**figure 3L**). Pro-caspase 9 intensity began to decrease as soon as after 8-hour treatment with βCCB, the cleaved caspase 9 being apparent from 16 hours. This caspase 9 activation was followed by a decrease in the pro-caspase 3 signal after 16 hours. A decrease in pro-caspase 8 was also observed, but only at the end of the assay time course, *i.e.* after 24 hours of treatment of the CGNs with βCCB.

We next addressed the question to know whether caspase activation was responsible for βCCB-induced CGN death by looking for a potential protective effect of caspase inhibitors. F**igure 4** shows that 20 µM BOC-D-FMK, a pancaspase inhibitor, rescued 67.0 ± 6.7% of

30 µM βCCB-treated neurons (mean ± SEM, n = 16; $P < 0.01$ ANOVA followed by Dunnett's post-tests). A significant protective effect against 30 µM βCCB-induced CGN death was also observed upon treatment with 20 µM Z-LEHD-FMK, a selective caspase 9 inhibitor (59.5 ± 16.2% of rescued cells; n = 16; $P < 0.01$ ANOVA followed by Dunnett's post-tests) and with 20 µM Z-DEVD-FMK, a selective inhibitor of caspase 3 (83.1 ± 16.0%; n = 15; $P < 0.05$ ANOVA followed by Dunnett's post-tests). Consistent with the absence of caspase 8 activation in βCCB-treated cultures, no significant increase in CGN survival was observed in the presence of 20 µM Z-IETD-FMK, a selective caspase 8 inhibitor (8 ± 5%; n = 7; $P > 0.05$; ANOVA followed by Dunnett's post-tests).

βCCB induces the release of mitochondrial proapoptotic factors without $\Delta\Psi_m$ loss

Since both immunofluorescence and immunoblotting experiments showed the prime activation of caspase 9 and since a caspase 9 inhibitor protected cultured CGNs against βCCB-induced death, this strongly suggests the involvement of the intrinsic (mitochondrion-triggered) apoptotic pathway. Therefore, we looked for the release of mitochondrial proapoptotic factors.

Ten to 12 hours after the beginning of the treatment with 30 µM βCCB, CGN cultures were fixed, immunolabeled for cytochrome c and stained with mitotracker red. As shown in **figure 5**, while punctated in control conditions, cytochrome c labelling of CGNs adopted a much more diffuse pattern upon βCCB treatment. Moreover, in βCCB-treated cells, cytochrome c staining did not co-localize anymore with mitotracker labelling, a marker of mitochondria, further suggesting that cytochrome c was released into the cytosol. Actually,

71.3 ± 9.7 % of βCCB-treated cells had such a diffuse mitotracker-distinct cytochrome c labelling compared to 14.5 ± 1.5 % in control cultures (n = 6; $P < 0.001$, unpaired *t*-test; **figure 5**).

In a same experimental paradigm (12 hours treatment with 30 µM βCCB), we looked for the location of AIF staining. **Figure 6** shows that AIF staining is mainly cytoplasmic in control conditions (**figure 6B**) and that it rarely and barely co-localizes with ethidium homodimer-1, a nuclear marker (**figure 6C**). On the opposite, in βCCB-treated cultures, AIF is predominantly located within the nucleus (**figure 6F**) where it moreover often co-localize with ethidium homodimer-1(**figure 6G**), likely reflecting its nuclear translocation.

The release of mitochondrial proapoptotic factors results from a permeabilization of the mitochondrial membrane. This permeabilization can either result from the formation of channels by pro-apoptotic Bcl-2 family members or from the formation of the mitochondrial permeability transition pore (mPTP)(Zamzami et al., 2001). The former mechanism only implies the outer mitochondrial membrane and does not affect the mitochondrial membrane potential ($\Delta\Psi_m$), while mPTP formation leads well to $\Delta\Psi_m$ loss. To get insight into the mechanism involved in our model, we monitored the $\Delta\Psi_m$ of 30 µM βCCB-exposed CGNs. As shown in **figure 7C**, no significant change in the green/red ratio of fluorescence emission of the JC-1 dye, as assessed by confocal laser microscopy, was observed after 8- to 12-hour 30 µM βCCB treatment of cultured CGNs ($n \approx 40$; $P > 0.05$, ANOVA followed by Dunnett's post-tests). These results were confirmed by fluorescence-activated cell sorting. **Figures 7A** and **7B** illustrate that the amount of βCCB-treated CGNs with a predominant FL2 emission (upper left quadrant), which reflects a high $\Delta\Psi_m$ and, hence, a normal mitochondrial membrane potential, was comparable to controls (79.5 ± 14.7 % in controls compared to 71.8

± 24.5 % in CGNs exposed for 12 hours to 30 µM βCCB; n = 2 experiments ; $P > 0.05$, unpaired t-test)

To confirm that the release of mitochondrial proapoptotic factors was independent of any $\Delta\Psi_m$ loss, and hence of mPTP opening, the effect of bongkrekic acid (BA) and cyclosporine A (CyA) on the survival of 30 µM βCCB-treated CGNs was assessed. Indeed, both BA and CyA are inhibitors of the mPTP and, thus, should not provide any significant protection if βCCB-induced neuronal apoptosis is truly mPTP-independent. As shown in **figure 7D** (open bars), neither 2 µM BA nor 5 µM CyA did provide any significant protection against 30 µM βCCB-induced toxicity towards cultured CGNs (n = 2 experiments ; $P > 0.05$; ANOVA followed by Dunnett's post-tests). These BA and CyA concentrations were however protective against 15 µM H_2O_2-induced toxicity in sister CGN cultures (filled bars in **figure 7D**). Consistently, neither CyA nor BA was able to oppose the release of cytochrome c induced by βCCB (data not shown).

Discussion

We had previously shown that two βCs, n-butyl-β-carboline-3-carboxylate (βCCB) and n-methyl-β-carboline-3-carboxamide (FG7142), induce the apoptotic cell death of cultured CGNs (Malgrange et al., 1996). However, the downstream events leading to apoptosis upon βCCB exposure of CGNs remained unknown. Here, we show that βCCB-induced death i) is neuron-specific, ii) is accompanied by and depends upon caspase 9 and caspase 3 activation, and iii) implies the release of proapoptotic factors, *i.e.* cytochrome c and AIF, without a disruption of the mitochondrial membrane potential.

At least part of the neuronal specificity of βCCB-induced cell death could be explained by our observation of the intracellular accumulation of βCCB in neurons, but not in astrocytes. Other likely explanations include differences in the apoptotic and/or cell defence machinery between both cell types. Such differences have been described for cultured astrocytes and neurons exposed to various noxious stimuli, *e.g.* NO, zinc or excitotoxins (Almeida et al., 2001; Sandhu et al., 2003; Dineley et al., 2000).

The pattern of caspase activation that we have observed strongly suggests that βCCB triggers the intrinsic pathway of apoptosis in cultured CGNs. First, caspase 9 is activated early in the βCCB-induced apoptotic process, well before the first signs of cell death and before the activation of caspase 3 or caspase 8 (see also below). Second, caspases 9 and 3 are indeed actual key players in the death of CGNs induced by βCCB, since inhibition of one of these two caspases by selective inhibitors provides a significant neuroprotective effect. We also observed an activation of caspase 8, but this occurred late in the βCCB-induced apoptotic process, *i.e.* after 24 hours only. This might suggest that caspase 8 activation would be a secondary downstream event, maybe consecutive to caspase 3 activation, as already described

in various models(Vaux et al., 1996). This hypothesis of a late, secondary, activation of caspase 8 was further confirmed by the lack of protection provided by caspase 8 inhibition. Because the activation of caspase 9 usually follows the release of cytochrome c from mitochondria, we looked for such a release. Our immunocytochemical results demonstrate that βCCB induces not only a cytosolic leakage of cytochrome c, but also a translocation of AIF into the nucleus. These results raise the question of how these mitochondrial factors are released. To address this issue, the $\Delta\psi_m$ was monitored during exposure of CGNs to βCCB and the effect of two mPTP inhibitors, *i.e.* CsA and BA, was tested. The absence of any $\Delta\psi_m$ loss and the lack of neuroprotective effect provided by addition of mPTP inhibitors suggest that the inner mitochondrial membrane (IMM) remains intact in dying cells following treatment with βCCB.. In such a case, the leakage of mitochondrial pro-apoptotic factors is usually thought to result from a selective permeabilization of the outer mitochondrial membrane (OMM) (Green et al., 1998). Channels formed by oligomerization of pro-apoptotic Bcl-2 family members, *e.g.* Bid, Bax, Bak, have been shown to be responsible for such a permeabilization in liposomes (Shimizu et al., 1999) and eukaryotic cells (Eskes et al., 2000; Eskes et al., 1998) leading to apoptosis in different experimental paradigms. For example, the importance of Bax was recognized in neuronal apoptosis induced by growth factor deprivation (Deckwerth et al., 1996). Furthermore, and more interestingly, a cytosolic release of cytochrome c without any $\Delta\psi_m$ loss has previously been demonstrated in cultured CGNs following exposure to MPP+ (Gonzalez-Polo et al., 2003). Since mPTP opening have been shown to be only transient in some circumstances (Szalai et al., 1999) and since our $\Delta\psi_m$ monitoring was only intermittent, one could argue that we could have missed mPTP openings and, hence, that we cannot completely rule out that it participates in βCCB-triggered apoptosis. However, if this were to be true, we would have expected some protective effect

from either CyA or BA. In our hands, both CyA and BA failed to rescue CGNs from βCCB-induced death while they protected them efficiently against H_2O_2-induced toxicity. This allows us to conclude that mPTP formation is very unlikely implied in βCCB-induced CGN apoptosis.

AIF is a caspase-independent apoptosis effector which, when released from mitochondria, translocates into the nucleus where it cleaves DNA into fragments of ~50 kpbs (Cande et al., 2002). The description of a nuclear translocation of AIF in our model should first be considered as an additional argument for a mitochondrial implication. The mechanism of mitochondrial AIF release remains unclear. Bax-induced mitochondrial membrane permeabilization seems not to be sufficient by itself (Arnoult et al., 2002) and some evidence suggests that AIF translocation, like cytochrome c release, may also occur independently of any $\Delta\psi_m$ loss (Arnoult et al., 2003). Nevertheless, our observed βCCB-induced AIF release probably explains why protection provided by caspase inhibitors was only partial.

The likely implication of the mitochondrial apoptotic pathway in βCCB-induced neuronal death together with the arguments in favour of a selective outer mitochondrial membrane permeabilization finally ask the question of the molecular target/receptor that triggers apoptosis. Since βCCB is a negative allosteric modulator at $GABA_A$ receptors and since we had already reported a significant protection against βCCB-induced death by GABA and benzodiazepines, a $GABA_A$ receptor-related hypothesis had been proposed in our previous study(Malgrange et al., 1996). Such a simple hypothesis now seems however unlikely. At least blockade of the $GABA_A$ receptor by itself does not induce neuronal death since a 24-hour treatment of CGNs with 10 μM bicuculline, a specific $GABA_A$ receptor antagonist, is devoid of any effect on their survival (data not shown). Furthermore, GABA and benzodiazepines have been shown to also provide protection against $GABA_A$ receptor-

unrelated insults, such as oxygen and glucose deprivation (Galeffi et al., 2000). The protective effect of GABA towards βCCB-induced toxicity could rather be related to its depolarizing effect on the membrane potential of cultured CGNs (Labrakakis et al., 1997) and the subsequent trophic effect, as already demonstrated for other depolarizing agents, *e.g.* high extracellular potassium(Alavez et al., 2003). An alternative hypothesis for the receptor of βCCB that triggers apoptosis includes the mitochondrial peripheral benzodiazepine receptor (PBR). This intracellular receptor is indeed thought to regulate the mitochondrial respiration and the free radical production, both events that are related to mitochondrion-triggered cell death(Beurdeley-Thomas et al., 2000). Moreover, the PBR has often been implied in cell death events(Casellas et al., 2002). This hypothesis is currently under investigation.

Nevertheless, to our knowledge, this study is so far the first attempt at deciphering the molecular events following treatment of cultured cells with βCCB. Conversely, the toxicity of MPP+, which is structurally close to βCCB, has been widely studied. Interestingly, an accumulation of MPP+ similar to that of βCCB has also been observed in cultured CGNs (Shang et al., 2003). Furthermore, after accumulating into neurons, MPP+, like βCCB, can induce a cytosolic cytochrome c release without any preceding $\Delta\psi_m$ loss and activate the same caspases, namely caspase 3 (Du et al., 1997; Bilsland et al., 2002) and caspase 9 (Han et al., 2003), although other studies suggested a non-apoptotic pathway.

In conclusion, the data presented here provide strong evidence that βCCB-induced CGN apoptosis is triggered by the mitochondria and involves the intrinsic pathway. This apoptotic neuronal death does not seem to imply the mPTP, at least during the first stages. However, further investigations are needed to identify both the intracellular receptor for βCCB and the precise mechanism leading to OMM permeabilization upon βCCB treatment. Participation of pore-forming BCL2 family members and VDAC are likely hypotheses.

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Figure legends

Figure 1 – βCCB induces a dose-dependent cell death in cultured CGNs

CGNs (●; filled circles) and astrocytes (○; open circles) were cultured for 24 hours in serum-free conditions and in the presence of increasing βCCB concentrations. Cell survival was determined using the MTT assay. Results are expressed as percentage of survival in control conditions, *i.e.* without added βCCB (mean ± SEM; n = 4 (astrocytes) and 8 (CGNs) replicate wells for each condition). The same results were obtained in at least two separate experiments.

* $P < 0.05$ and ** $P < 0.01$ using ANOVA followed by Dunnett's multiple comparison post-tests.

Figure 2 – Neuron-specific intracellular accumulation of [^{3}H]βCCB

A Binding/uptake isotherm of [^{3}H]βCCB in cultured CGNs. [^{3}H]βCCB specifically bound (dpm corrected for protein content) to cultured CGNs is plotted against free [^{3}H]βCCB concentration (AI = arbitrary units, *i.e.* 1:100,000 dilution of the stock solution). *Inset*: Scatchard transformation of data in **A**.

B Effects of increasing concentrations of βCCB on [^{3}H]βCCB (1:20,000 dilution of the stock solution) specific binding/uptake in cultured CGNs. Results are expressed as percentage of [^{3}H]βCCB specific binding/uptake in control buffer (mean ± SD; n = 3 for each condition. Data were fitted with a one (dashed line) or two sites (plain line) competition curve.

C Dissociation/release kinetics of [^{3}H]βCCB from cultured cells. [^{3}H]βCCB specifically bound to cultured CGNs (●; filled circles), astrocytes (○; open circles) and 3T3 fibroblasts (Δ; open triangles) (dpm corrected for protein content; 1:20,000 dilution of the stock solution) is expressed as a function of washing time. For CGNs and astrocytes, data were fitted with a single exponential decaying curve.

D As in **C**, dissociation/release kinetics of [^{3}H]βCCB from cultured CGNs in normal (5.4 mM; Δ; open triangles) and high (25 mM; ▲; filled triangles) extracellular potassium.

Figure 3 – Activation of caspases in βCCB-treated CGNs

LEFT Confocal micrographs illustrating the activity of several caspases assessed by using specific carboxyfluorescein-labelled caspase inhibitors (FAM-LETD-FMK for caspase 8, FAM-LEHD-FMK for caspase 9 and FAM-DEVD-FMK for caspase 3) in 12-hour-old cultured CGNs counterstained with ethidium homodimer (red). **A**, **C** and **E**: 30 μM βCCB-treated cultures. **B**, **D** and **F**: control cultures. Bar = 12.5 μM for all pictures.

CENTER The graphs illustrate the activation of the different caspases (**G**: caspase 3; **H**: caspase 9 and **I**: caspase 8) 10 and 12 hours after exposure to 30 μM βCCB (filled bars) or in control conditions (empty bars). Results are expressed in percentage of cells presenting a caspase activation (mean ± SD; for n, see text).

*** $P < 0.001$ using ANOVA followed by Dunnett's multiple comparison post-tests.

RIGHT Immunoblot analysis of caspase-9, -3 and -8 at four different times after treatment of CGN cultures with 30 μM βCCB. A reduction of the pro-caspase band and/or

the apparition of a cleaved fragment indicate the activation of the corresponding enzyme (**K**). The band intensities were analysed by using the ImageMaster 1D software and were expressed as percentage of the intensity in controls, *i.e.* before addition of βCCB (**L**).

Figure 4 – Some caspase inhibitors protect CGNs against βCCB-induced death

CGNs were cultured for 24 hours in serum-free conditions and in the presence of 30 µM βCCB alone or together with 20 µM BOC-D-FMK, a pancaspase inhibitor, Z-LEHD-FMK, a caspase 9 inhibitor, Z-DEVD-FMK, a casapse 3 inhibitor or Z-IETD-FMK, a caspase 8 inhibitor. Cell survival was determined using the MTT assay. Results were normalized so that 100% represent the survival in control conditions and 0% the survival in the presence of 30 µM βCCB (mean ± SEM; n = 7-35).

** $P < 0.01$ using ANOVA followed by Dunnett's multiple comparison post-tests.

Figure 5 – βCCB induces a cytosolic release of cytochrome c in cultured CGNs

LEFT Confocal micrographs after labelling cytochrome c (green) and mitotracker (red) in control (above picture) and in 30 µM βCCB-treated CGN cultures (below picture).

Bar = 5 µM for both pictures.

RIGHT Proportion of CGNs presenting a cytosolic release of cytochrome c in βCCB-

treated CGNs compared to controls. Results are expressed as percentage of total cells (mean ± SEM; n = 5-6).

*** $P < 0.001$ using Student *t*-test.

Figure 6 – βCCB induces a nuclear translocation of AIF in cultured CGNs

Confocal micrographs after labelling ethidium homodimer-1 (Etd1; red) and apoptosis-inducing factor (AIF; green) in controls (**A** to **C**) and in 30 µM βCCB-treated CGN cultures (**E** to **G**). Note the condensed labelling of Etd1 in **E** compared to **A**, together with its co-localization with AIF (**G**).

Bar = 12.5 µM for all pictures.

Figure 7 – Absence of mitochondrial membrane depolarisation in βCCB-treated CGNs

TOP CGNs were cultured for 12 hours in serum-free conditions in the absence (controls; **A**) and in the presence of 30 µM βCCB (**B**), loaded with JC-1 and subsequently subjected to cytofluorometric analysis. Emission in FL1 (abscissas) corresponds to JC-1 monomers and in FL2 (ordinates) to aggregates.

C JC-1 emission ratios (590/530 nm) were measured in control cultured CGNs (yielding 100%) and 8, 10 and 12 hours after treatment with 30 µM βCCB. $P > 0.05$ using ANOVA.

D The survival of cultured CGNs treated for 24 hours by 30 µM βCCB (open bars) or by 15 µM H_2O_2 (filled bars) was measured in the absence and in the presence of 5 µM cyclosporine A (CyA) or of 2 µM bongkrekic acid (BA). Results are expressed as in

figure 1. $P < 0.01$ using ANOVA followed by Dunnett's post-tests.

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Figure 1

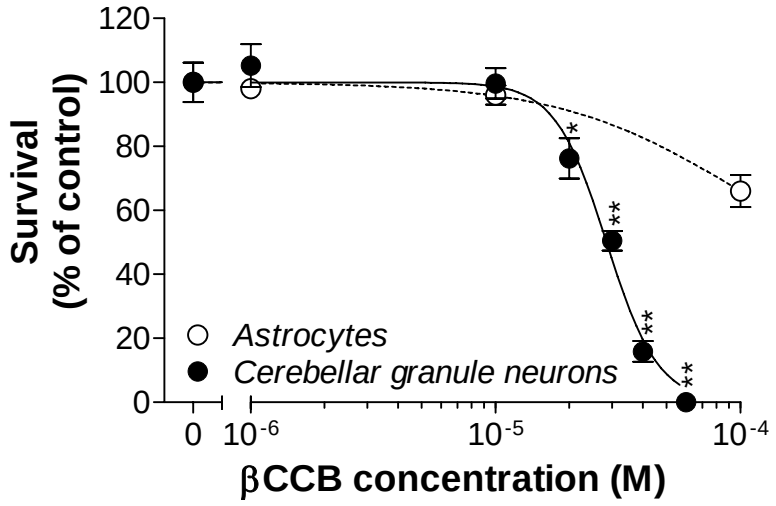

Survival
(% of control)
120
100
80
60
40
20
0
Astrocytes
Cerebellar granule neurons
**
**
**
**
0
10-6
10-5
10-4
βCCB concentration (M)

Figure 2

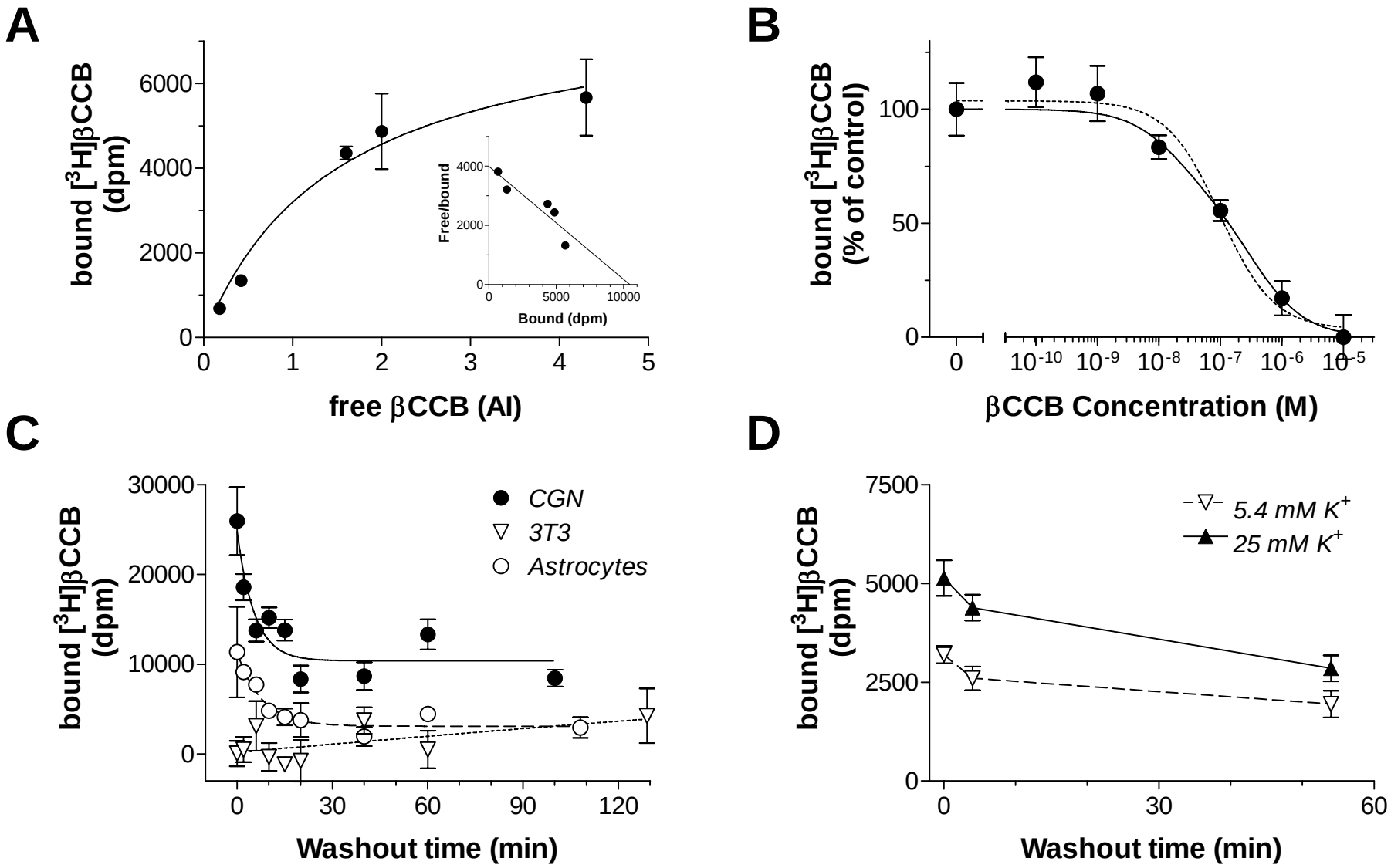

A
bound [3H]βCCB (dpm)
6000
4000
2000
0
0
1
2
3
4
5
free βCCB (AI)
Free/bound
4000
2000
0
0
5000
10000
Bound (dpm)
B
bound [3H]βCCB (% of control)
100
50
0
0
10-10
10-9
10-8
10-7
10-6
10-5
βCCB Concentration (M)
C
bound [3H]βCCB (dpm)
30000
20000
10000
0
CGN
3T3
Astrocytes
0
30
60
90
120
Washout time (min)
D
bound [3H]βCCB (dpm)
7500
5000
2500
0
5.4 mM K+
25 mM K+
0
30
60
Washout time (min)

Figure 3

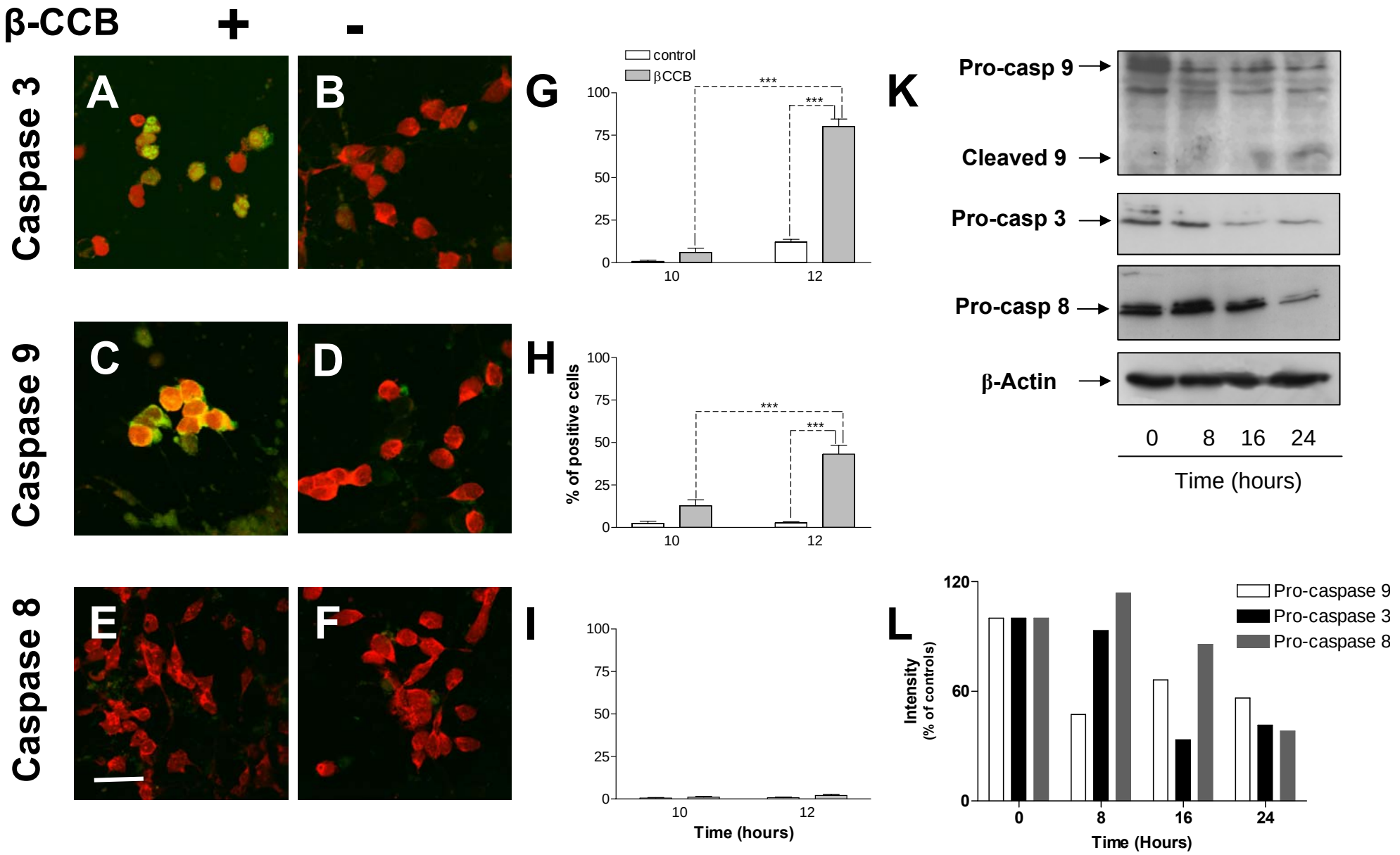

β-CCB
+
-
Caspase 3
Caspase 9
Caspase 8
A
B
C
D
E
F
G
control
βCCB
***
***
100
75
50
25
0
10
12
H
% of positive cells
I
Time (hours)
K
Pro-casp 9
Cleaved 9
Pro-casp 3
Pro-casp 8
β-Actin
0
8
16
24
Time (hours)
L
120
60
0
Intensity
(% of controls)
Pro-caspase 9
Pro-caspase 3
Pro-caspase 8
Time (Hours)

Figure 4

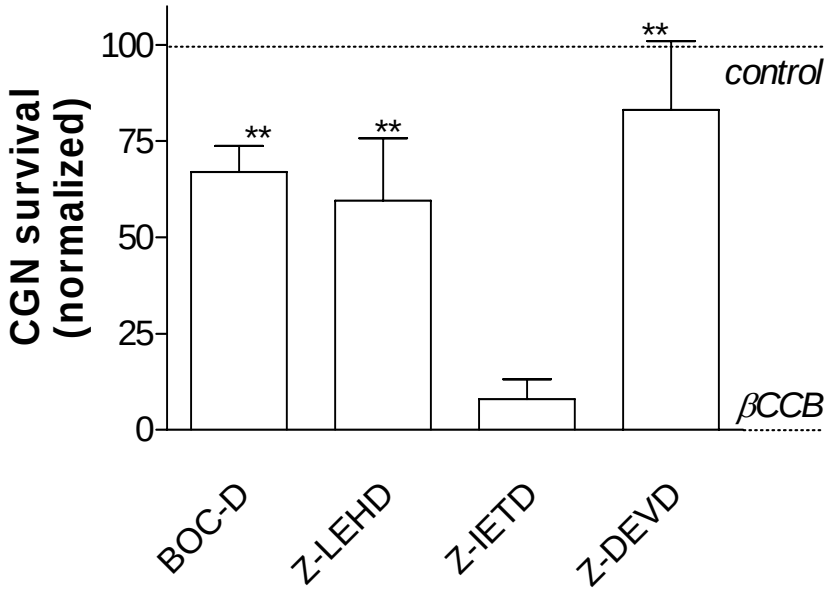
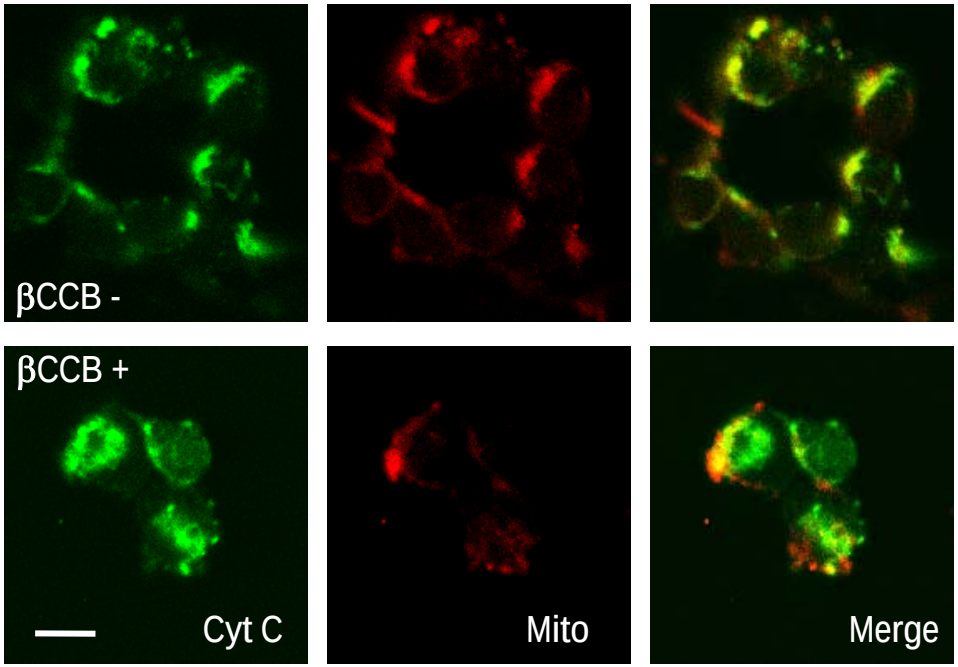

CGN survival
(normalized)
100
75
50
25
0
control
βCCB
**
**
**
BOC-D
Z-LEHD
Z-IETD
Z-DEVD

Figure 5


βCCB -
βCCB +
Cyt C
Mito
Merge

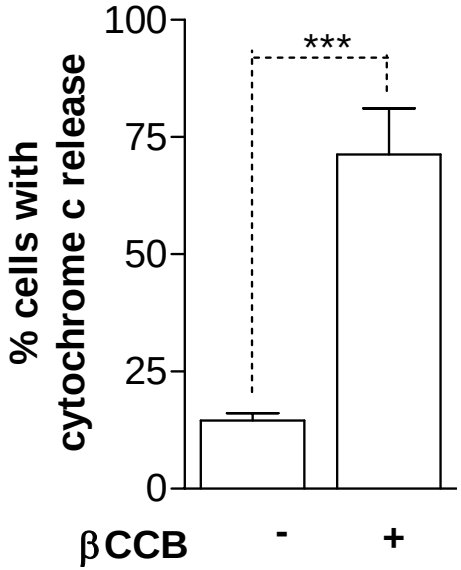

% cells with cytochrome c release
100
75
50
25
0
***
βCCB
-
+

Figure 6

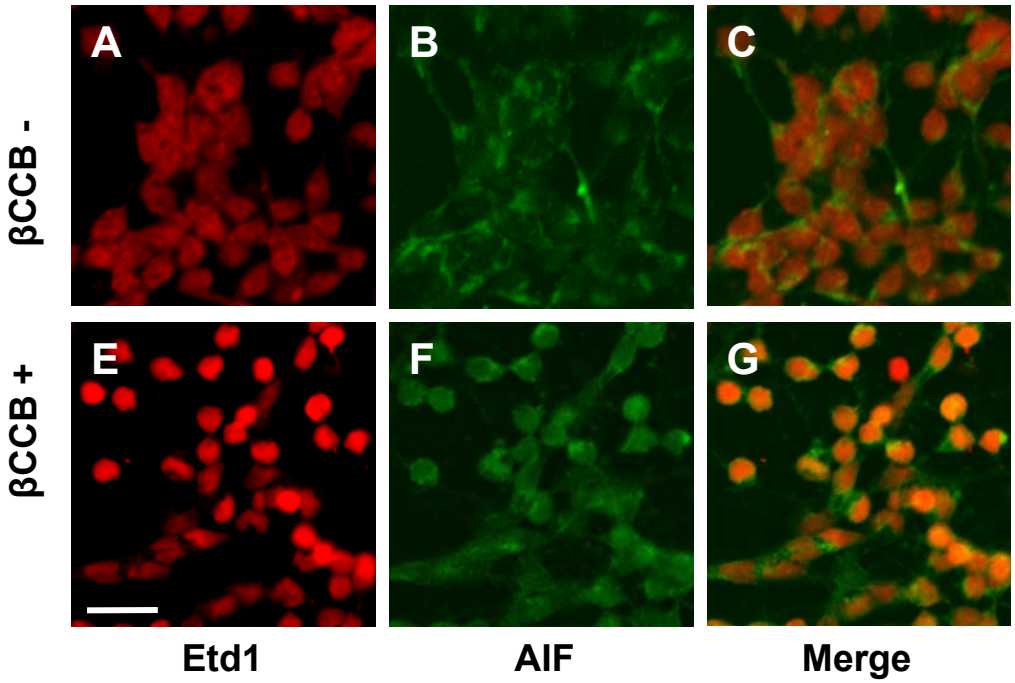
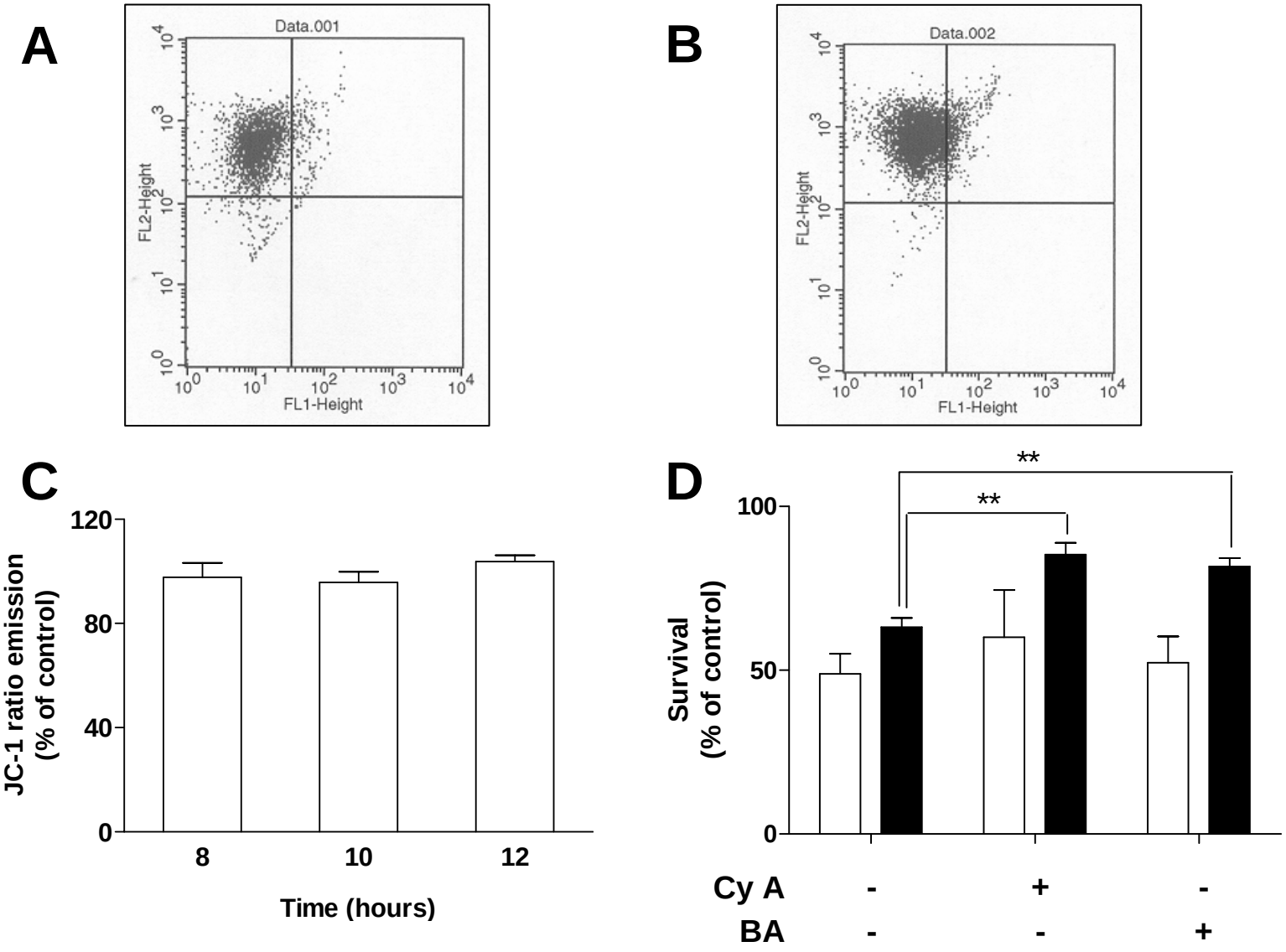


Figure 7


A
Data.001
FL2-Height
FL1-Height
B
Data.002
FL2-Height
FL1-Height
C
JC-1 ratio emission
(% of control)
120
80
40
0
8
10
12
Time (hours)
D
**
**
Survival
(% of control)
100
50
0
Cy A - + -
BA - - +