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Endogenous XIAP but not other members of the inhibitory apoptosis protein family modulates cerebellar granule neurons survival

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Running Title: XIAP levels control CGN survival

ABSTRACT

Inhibitor of apoptosis proteins (IAPs) are implicated in negative regulation of caspases and cell death. Here, the role of some members of IAPs in the neuronal survival was studied under physiological conditions. We found a differential expression pattern of cIAP-1, cIAP-2, XIAP and survivin during cerebellar development in an age-dependent manner, highlighting the significant increase of XIAP levels. We also detected an interaction between XIAP and caspase 3 at postnatal day (P) 12 and 16. On the other hand, we found a significant decrease of XIAP levels in cultured cerebellar granule neurons (CGN) maintained in chronic potassium deprivation, an apoptotic condition, suggesting a possible relationship between XIAP levels and neuronal viability. Under these conditions, we also detected the interaction of XIAP with active caspase-3. The down-regulation of XIAP ~~down-regulation of~~ in CGN cultured under survival conditions (chronic potassium depolarization) induced a reduction of cell viability and an increment of apoptotic cells. These findings support the idea that IAPs could be involved in the survival of CGN and that XIAP might be critical for neuronal survival promoted by chronic depolarization through a mechanism involving caspase inhibition.

Key words: apoptotic-like death, IAPs, caspases, cerebellum development, cerebellar granule neurons, potassium deprivation.

1. Introduction

Programmed cell death (PCD) is a physiologic and active process involved in organogenesis during development. PCD has a critical role in the development of the nervous system and their modulators act prominently in the establishment of neural architecture. In several pathological disorders the deregulation of PCD leads to developmental and neoplastic alterations of the nervous system (for review, see Bredesen et al., 2006). Apoptosis is a type of PCD that plays an important role during maturation of the central nervous system (CNS). Apoptosis can be defined by a series of morphological changes, including chromatin condensation, cytoplasmic shrinkage, zeiosis and the formation of apoptotic bodies, which is accompanied by a phosphatidylserine translocation. In the most classic form of apoptosis, it is observed the activation of caspases (aspartate-specific cysteine proteases) (for review, see Leist and Jättelä, 2001).

Caspases are negatively regulated by ~~the~~ inhibitor of apoptosis proteins (IAPs). This is a family of proteins comprised by NAIP, cIAP-1/hIAP-2/MIHB, cIAP-2/hIAP-1/MIHC, XIAP/hILP-1/MIHA, survivina/TIAP, ILP-2/Ts-IAP, ML-IAP/Livin/KIAP and Bruce/Apollon (Rothe et al., 1995; Roy et al., 1995; Duckett et al., 1996; Liston et al., 1996; Ambrosini et al., 1997; Hauser et al., 1998; Chen et al., 1999; Vucic et al., 2000; Richter et al., 2001). There are several evidences showing the importance of IAPs in the neuronal protection against damage and in neuronal survival promoted by trophic support. For example, the avian homologue of cIAP-2 is essential for the neuronal survival promoted by neuronal growth factor (NGF) in sensorial and sympathetic neurons in culture (Wiese et al., 1999). Similarly, XIAP and NAIP are critical for the protective

effect of glial cell-derived neurotrophic factor (GDNF) observed in motoneurons (Perrelet et al., 2002). Additionally, the elimination of XIAP is sufficient to activate caspases in the presence of cytosolic cytochrome c in sympathetic neurons (Potts et al., 2003). In cultured hippocampal neurons, the treatment with low concentration of *N*-methyl-D-aspartate (NMDA) increases XIAP levels and inhibits ~~the~~ caspase-3 activation. These neurons are more resistant to die by action of glutamate and staurosporine (Korhonen et al., 2004).

During cerebellar development, the external granule layer (EGL) gives rise to cerebellar granule neurons (CGN) (Ito, 1984). In rat, CGN proliferate in the EGL during the first two postnatal weeks (Altman, 1972). Postmitotic immature CGN migrate through the molecular layer (ML) into the internal granule layer (IGL) between postnatal day 3 (P3) and P21 (Burgoyne and Cambray-Deakin, 1988). Once in the IGL, CGN begin to receive ~~start receiving~~ excitatory synaptic contacts from mossy fibers at around P5, and most of these synaptic contacts are already functional from P10 to P12 (Arsenio-Nuñez and Sotelo, 1985; Burgoyne and Cambray-Deakin, 1988). Along this process a considerable number of CGN die (Koppel et al., 1983) with characteristics of apoptotic death (Wood et al., 1993; Alavez et al., 2006a, 2006b). It has been suggested, that the mossy fibers might exert a trophic action on CGN during cerebellar development. This hypothesis is supported by the trophic effect exerted by potassium depolarization or the stimulation of NMDA receptors (NMDAR) of cultured CGN that could be mimicking the trophic influences of mossy fiber afferents on CGN during *in vivo* development (Gallo et al., 1987; Balàzs et al., 1988a, 1988b).

Several studies have shown that CGN cultured under physiological concentrations of potassium (5 mM KCl; K5) during more than 5 days *in vitro* (DIV) die by apoptosis ~~apoptotically~~ (Alavez et al., 2003; Xifro et al., 2005). In contrast, the survival and differentiation of these cells in culture is markedly increased in the presence of high concentrations of potassium (25 mM KCl; K25) (Gallo et al., 1987; Balàzs et al., 1988b; Morán and Patel, 1989a, 1989b; Xifro et al., 2005) or NMDAR stimulation (Balàzs et al., 1988a, 1988b; Morán and Patel, 1989b; Xifro et al., 2005). During the last decade, cultured CGN have become a widely used model for the study of PCD (D'Mello et al., 1993; Morán et al., 1999; Alavez et al., 2003; Xifro et al., 2005). In this preparation, it has also been examined the endogenous expression of IAPs. Recently, we have found a basal expression of cIAP-1, cIAP-2, XIAP and survivin, but not of BRUCE (Maycotte et al., 2008). Moreover, it has been described the interaction between caspase-3 and XIAP in cultures of CGN (Xifro et al, 2006). In addition, there are studies showing a protective effect of the overexpression of IAPs in cultures of mature ~~cultured~~ CGN. The overexpression of human XIAP, cIAP-1, cIAP-2 or NAIP induces a delay in the apoptotic death of CGN induced by K5 (Simons et al, 1999; Eldadah et al, 2000), but not by excitotoxic stimuli (Simons et al, 1999)

In particular, there is no experimental evidence showing the role of IAPs in the programmed death of CGN during cerebellar development *in vivo* or in cultured CGN under conditions that mimic the developmental process. In the present work, we studied the role of IAPs in the mechanisms of CGN survival when cells are cultured chronically in K25 or K5 and during postnatal cerebellar

development in an attempt to evaluate the participation of IAPs during CGN death/survival in the cerebellar development.

2. Experimental procedure

2.1 Animals and cell culture

Wistar rat pups at postnatal day 0, 4, 8, 12, 16 and 25 (P0 to P25, respectively) were obtained from the animal house at the Instituto de Fisiología Celular. Animals used for experimentation in this study were treated in accordance with procedures approved by the local Committee of Research and Ethics of the Instituto de Fisiología Celular, Universidad Nacional Autónoma de México. Protocols used followed the Guidelines for the Use of Animals in Neuroscience Research as well as the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

CGN were obtained as previously described (Morán and Patel, 1989a). Briefly, dissociated cell suspensions of 8-day-old Wistar rats cerebella were plated at a density of 265×10^3 cells/cm^2 in plastic dishes coated previously with poly-L-lysine (5 μg/ml) or in plastic dishes with coverslips using poly-L-lysine 25 μM. Culture medium contained basal Eagle's medium supplemented with 10% (v/v) heat inactivated fetal calf serum, 2 mM glutamine, 50 μg/ml streptomycin and 50 U/ml penicillin. Culture dishes were incubated at 37 ºC in a humidified 5% CO_2/ 95% air atmosphere, and cytosine arabinoside (10 μM) was added after 18 hr. CGN were grown chronically on low (5 mM KCl; K5) or high potassium (25 mM KCl; K25). In some experiments cells were grown for two

days in K5 and then cultures were treated with K25 and cells were harvested for their analysis at different days *in vitro* (DIV).

2.2 Cell viability

In vitro cell viability was estimated by using MTT assay (Mossman, 1983). After treatment, cells were incubated with MTT (40 μg/ml) for 15 min at 37 °C and after medium removal, formed formazan blue was extracted with DMSO and quantified spectrophotometrically at 570 nm excitation wavelength.

2.3 Immunoblots

Cells were washed with PBS and homogenized with lysis buffer (Tris 25 mM, NaCl 50 mM, Igepal 2%, SDS 0.2% and Complete protease inhibitors (Roche), pH 7.4). Pups at different ages (P0-P25) were anesthetized with pentobarbital and decapitated. The cerebellum was dissected at 4 °C and cerebella were washed with PBS and homogenized with lysis buffer. Protein concentration was quantified by the method of Bradford (Bradford, 1976). Homogenates were separated in SDS-PAGE gels (12 or 15%) and transferred to PVDF membranes (Millipore, Immobilon). Blots were blocked with Tris-buffered saline (TBS; 100 mM Trizma, 150 mM NaCl, pH 7.5) containing 5% nonfat dry milk for 1 h, washed with TTBS (TBS with 0.1% Tween) and incubated overnight at 4 °C with the primary antibody raised against cIAP-2 (1:100 dilution, Santa Cruz Biotechnology, Inc.); cIAP-1 (1:100 dilution, Santa Cruz Biotechnology, Inc.); XIAP (1:250 dilution, BD Biosciencies Transduction Lab.); survivin (1:1000 dilution, NOVUS Biologicals); caspase-3 (1:100 dilution, Santa Cruz Biotechnology, Inc) or GAPDH (1:2000 dilution, Millipore). After

washing, blots were incubated with a secondary antibody coupled to alkaline phosphatase (anti-rabbit: 1:30000 dilution, Zymed; anti-mouse: 1:30000 dilution, SIGMA) for 1 h at room temperature. Blots were processed for visualization using an enhanced chemiluminiscence system according to the manufacturer's recommendations (CDP-Star, BioLabs, Inc) and exposed to Kodak film. Densitometric analysis was done using the Image-J program, NIH Image.

2.4 Immunohistochemistry

Pups at different ages (P0-P25) were anesthetized with pentobarbital and perfused transcardially with PBS followed by 4% paraformaldehyde at 4 °C in 0.1 M phosphate buffer, pH 7.4. Whole brains were postfixed overnight in 4% paraformaldehyde at 4 °C, cryoprotected with increasing concentrations of sucrose (15 and 30%) in PBS, and cerebella were frozen in Tissue-Tek embedding medium (Sakura Finetek U.S.A., Inc.). Sagittal frozen sections (35 μm) were collected and stored at -20 °C until additional processing. Subsequently, sections were blocked and permeabilized in 10% normal goat serum and 0.5% Triton X-100 in PBS overnight at 4 ºC, followed by incubation with rabbit polyclonal antibodies against cIAP-2 (1:50 dilution, Santa Cruz Biotechnology, Inc.); XIAP (1:50 dilution, Cell Signaling) or survivin (1 μg/μl, NOVUS Biologicals) for two days at 4 ºC. The primary antibody was detected with a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody (1:250 dilution, ZIMED) incubation for 1 h at room temperature. Slides were mounted with especial medium for fluorescence with DAPI (Vectashield, Vector Laboratories, Inc). Sections were then examined under a confocal laser-scanning microscope (Olympus Fluoview FV1000).

2.5 Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from cerebellar granule neurons at 7DIV with Trizol accordingly to manufacturer's instructions. Total RNA (120 ng) was converted to first-strand cDNA using the SuperScript II Reverse Transcriptase (Invitrogen). The resulting cDNA was subjected to PCR analysis. PCR cycling parameters were as follows: 94°C for 2 min, followed by 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s for 30 cycles; 72°C for 5 min with *xiap* and *18S* primers. The PCR products were stained with SYBR Safe (Invitrogen). Samples without RNA, Reverse Transcriptase or Polymerase were used as negative control (data not shown). The sequences of specific primers are as follows: xiap mRNA, forward, TGAAGAAGCCAGACCGAAGA; reverse, TGACTTGACTCATCCTGCGA; and 18S, forward, TCAAGAACGAAAGTCGGAGG; reverse GGACATCTAAGGGCATCACA.

2.6 Immunoprecipitation

The interaction of XIAP with active caspase-3 was determined by immunoprecipitation assays previously described (Xifro et al., 2006). CGN were washed twice with PBS and lysed at 4 °C in a buffer with Tris-HCl 50 mM pH 7.5; EDTA 10 mM; Tritón X-100 1% and Complete protease inhibitors (Roche). Pups at P12 and P16 were anesthetized with pentobarbital and decapitated. The cerebellum was dissected at 4 °C, washed with PBS and lysed with the buffer above mentioned. Protein concentration was quantified by the method of Bradford (Bradford, 1976) and 1 mg of protein was incubated with 1 μg of mouse anti-XIAP antibody (BD Biosciencies Transduction Lab.) at 4 °C

overnight in rotation. Then, protein G-agarose was aggregated to each reaction and was incubated for 1h at 4 °C in rotation. The immune complex was recuperated by centrifugation at 800 rpm for 2 min at 4 °C and washed three times with buffer. The pellet was denatured with 3x standard loading buffer and the proteins were separated in SDS-PAGE gels (15%) and the detection of XIAP and caspase-3 active subunits was done by immunoblots as previously detailed.

2.7 Plasmids

For RNA interference (RNAi) experiments, constructs were generated in the pSUPER.retro.puro plasmid (OligoEngine) using specific oligonucleotides against rat XIAP (sequence data available from GeneBank under accession number NM_022231) indicated by capital letter, as follows: RNAi, forward, gatccccAGAATCCTATGGTGCAAGAttcaagagaTCTTGCACCATAGGATTCtttttt; reverse, agctaaaaaAGAATCCTATGGTGCAAGAtctcttgaaTCTTGCACCATAGGATTCggg. A scrambled sequence was used as a control by the following oligonucleotides: RNAi, forward, gatccccAGAACACGACGGAACAAGAttcaagagaTCTTGTTCCGTCGTGTTCTttttt; reverse, agctaaaaaAGAACACGACGGAACAAGAtctcttgaaTCTTGTTCCGTCGTGTTCTggg. Oligonucleotides were provided by Invitrogen and cloned between BglII and HindIII sites of the pSUPER.retro.puro vector, under the control of the DNA Pol III promoter of H1. Lentiviral constructs were achieved by digestion at the EcoRI-ClaI sites to replace H1 promoter in the lentiviral vector pLVTHM with

H1-short hairpin RNA cassette from pSUPER. The pLVTHM-H1-RNAi was used for lentiviral knockdown experiments.

2.8 Lentiviral production

Lentiviruses were propagated as described previously (Naldini, 1996; Zufferey, 1998). Briefly, human embryonic kidney cells (HEK293T) cells were seeded at 2.5×10^5 cells/ml in 100 mm dishes. At 24 hours, cells were transfected with 20 µg of pLVTHM- constructs, 15 µg of psPAX2 and 6 µg of pMD2.G using the calcium phosphate method (Cullen, 1987). After 48 hours, the medium was centrifuged at 25,000 rpm for 2h at 4ºC and pelleted viruses were resuspended in TNE buffer (50 mM Tris-HCl pH 7.5, 130 mM NaCl and 1 mM EDTA) by shaking at 175 rpm overnight at 4ºC. Lentiviruses were stored at -80ºC. Biological titers of the viral preparations expressed as a number of transducing units (TU) per millilitre were determined by infecting HEK293T cells with serial dilutions. After 48 hours, the percentage of GFP-positive cells was detected by flow cytometry (Cytomics FC 500, Beckman Coulter) and analyzed with RXT software.

2.9 Cell transfection and infection

Lentiviral infection was performed at the time cerebellar granule cells were seeded and medium was changed 5 hours after infection. The efficiency of infection in each experiment was monitored by direct counting of GFP-positive cells. The percentage of infected cells reached at least 75% in granule cultures. The efficiency of down-regulation of XIAP was assessed by immunobloting.

2.10 Statistical Analysis

Data are expressed as the mean +/- S.E.M. values. Two sample t Test or One-way ANOVA followed by Levene's test (for population variations) or ~~and~~ Tukey test~~s~~ (for mean comparison) was used. ~~for mean comparisons.~~

3. Results

3.1 IAPs levels and localization in the cerebellar cortex during cerebellar development

We measured the levels of some IAPs in cerebella during postnatal development of rats. cIAP-1, cIAP-2, XIAP and survivin levels are detectable at all times of cerebellar postnatal development but with differential expression patterns (Fig. 1). cIAP-1 levels did not show changes during cerebellar development (Fig. 1A), but cIAP-2 presented a clear tendency to decrease with age and was statistically reduced from ~~form~~ P12, P16 and P25 compared to P0, P4 and P8, respectively (Fig. 1B). XIAP levels statistically increased at P16-25 compared to P0 and P8 levels (Fig. 1C). Survivin levels statistically decreased at P25 compared to previous ages (Fig. 1D). We confirmed the changes of levels of cIAP-2, XIAP and survivin in the cerebellar cortex by immunohistochemistry of cerebellar sections taken at different postnatal days (Fig. 2). Under these conditions, we found changes of IAPs levels in the cerebellar cortex in an age-dependent manner. cIAP-2 and survivin showed a decrease at P16 compared to P4, conversely XIAP increased at P16 compared to P8 (Fig. 2).

3.2 Effect of K5 in IAPs levels and viability of cultured CGN

We measured the levels of IAPs in CGN cultured in K5 or K25 during different days *in vitro* (DIV). cIAP-1, cIAP-2, XIAP and survivin levels are detectable at all DIV and under both condition, but with differential expression patterns (Fig. 3 and 4). The *cIAPs* mRNA levels did not show marked differences in CGN cultured in K5 or K25 by several days (Fig. 3A). This was consistent with protein levels of both cIAP-1 and cIAP-2 that did not show changes in CGN treated chronically with K5 or K25 at any DIV analyzed (Fig. 3B and C). However, both mRNA and protein levels of survivin decreased with time in culture (Fig. 3A and D). Particularly, the protein levels diminished at 7 DIV independently of culture condition, K5 or K25 (Fig. 3D).

On the other hand, the mRNA of XIAP did not change under any culture condition or DIV analyzed (Fig. 4A). In contrast, at 7 DIV, the protein levels decreased in K5, but not with K25 (Fig. 4B). When CGN were treated with K5 for 2 days and then cultured with K25 for 5 more days (K25-5d), XIAP levels were restored to control levels (Fig. 4B). At this time, 7 DIV, K25 promotes CGN survival (Fig. 4C), while K5 induces CGN death and under K25- 5d condition the viability was restored to control (Fig. 4C). Importantly, the XIAP levels at 7DIV seem to correlate with the CGN viability (Fig. 4B and C). These data suggest a central role of XIAP in CGN survival induced by K25.

3.3 Effect of K5 in XIAP-Caspase-3 interaction in cultured CGN and cerebellar development

In order to evaluate the participation of XIAP in neuronal survival by caspase-3 inhibition, we evaluated the XIAP/active caspase-3 interaction using

immunoprecipitation assays (Fig. 5). In CGN cultured for 6 DIV, the interaction of XIAP with active subunits of caspase-3 was observed in K5, K25 and in cells cultured 2 days in K5 and then treated with K25 for 4 more days (K25-4d) (Fig. 5A). Under these conditions, after the recuperation of the immune complex in the immunoprecipitation assays, we still observed free active subunits of caspase-3 in the supernatants of neurons cultured in K5, but not in K25 or K25-4d (Fig. 5A). These results suggest that under K5 conditions, the low levels of XIAP observed are insufficient to counteract the active caspase-3 present.

On the other hand, in the cerebellar homogenates we found an interaction of XIAP with caspase-3 at P12 and P16 (Fig. 5B). However, in the supernatant, at P12, a time when there is a high rate of granule neurons death in the cerebellum, we detected free active subunits of caspase-3, but not at P16, when cell death process is low (Fig. 5B).

3.4 Effect of XIAP inhibition on viability of cultured CGN

To confirm the significance of XIAP in granule neurons survival, we determined the effect of XIAP inhibition on CGN viability by using a RNAi against XIAP. We corroborated the RNAi effectiveness measuring the levels of mRNA and protein of XIAP (Fig. 6A and B), and transfection efficiency with the reporter GFP (Fig. 6C). The XIAP down-regulation induced an increment of apoptotic cells and a reduction of viability, measured as condensed nuclei and a reduction of MTT transformation, respectively (Fig. 6D and E). These data suggest that XIAP have a key role essential in maintaining CGN survival under chronic depolarization conditions.

4. Discussion

In the present work, we evaluated the cerebellar expression of cIAP-1, cIAP-2, survivin, and XIAP during development and confirmed their presence in the postnatal cerebellar cortex. These IAPs show differential expression patterns in an age-dependent manner. Thus, during cerebellar development there is a decrease of cIAP-2 and survivin and an increase of XIAP. The decrease of cIAP-2 levels in cerebellum from P12 could be related to the death process of CGN that occurs during cell migration (from EGL to IGL) and synaptogenesis of CGN (Koppel et al., 1983). On the other hand, the decrease of survivin levels at late cerebellar development could be associated with the decline of the cell proliferation of CGN, stellar and basket cells (Altman, 1972; Goldowitz and Hamre, 1998). Finally, the increment of XIAP levels at P16 could be related to the trophic effect of mossy fiber afferents on CGN (Gallo et al., 1987; Balàzs et al., 1988a, 1988b). These data suggest that the requirements of cIAP-2 and survivin are early in development and that XIAP is required later during cerebellar development, therefore XIAP could be an element of cellular survival in the fully developed cerebellum.

It is known that CGN chronically cultured with low concentrations of potassium (K5) die with apoptotic characteristics (Alavez et al., 2003; Xifro et al., 2005; Blancas and Moran, 2011), including nuclear condensation, phosphatidylserine translocation and caspases activation. On the other hand, chronic depolarizing concentrations of potassium (K25) promote CGN survival (Gallo et al., 1987; Balàzs et al., 1988b; Morán and Patel, 1989a, 1989b; Xifro et al., 2005). Similarly, there is a survival effect of K25 at 7 DIV when CGN were

cultured for two days in K5 and then in K25. We previously reported that CGN in K5 and rescued at 2 DIV by K25 show at 7 DIV a reduced number of condensed nuclei (Xifro et al., 2005) and do not have caspase-3 activity (Blancas and Moran, 2011), as well as chronic K25 condition. The possible role of IAPs family in CGN survival induced by K25 has not been elucidated. In this study, we corroborated the expression of cIAP-1, cIAP-2, XIAP and survivin in mature CGN cultured in depolarizant concentrations of potassium (Maycotte et al., 2008) and identified these IAPs in CGN cultured in chronic K5. There is a differential regulation of the IAPs induced by K5, thus K5 does not modify the protein levels of cIAPs and survivin from 2-7 DIV but decreases the XIAP levels at 7 DIV. Importantly, the cIAPs levels do not change by potassium concentration nor age of culture, whereas survivin levels do not change by potassium but decrease with culture age. Therefore cIAPs and survivin do not appear to be associated with CGN survival promoted by K25. We previously showed that death of mature CGN in culture induced by staurosporine go with decrease of cIAP-1 and cIAP-2 protein levels and with mitochondrial release of, the IAPs negative regulator, Smac to cytoplasm (Maycotte et al., 2008). Together, the data about cIAPs support the idea that cIAPs could be associated to neuronal survival according to neuronal mature status and could be differentially affected by diverse death stimuli. On the other hand, the survivin reduction observed in CGN *in vitro* is consistent with the decrease detected in the cerebellum during development. This suggests that the cellular requirements of survivin are relevant in the early cerebellar development and in CGN of young cultures (2-3 DIV) but are not important in granule neurons by their depolarizant condition.

As mentioned above, here we corroborated that CGN chronically cultured are mostly death in K5 at 7 DIV. We found a positive correlation between CGN survival and total XIAP levels, which showed a marked reduction by K5 at 7 DIV. According with this information we suggest that XIAP could be a survival factor in CGN chronically cultured in K5 during the first *in vitro* days, we have previously shown that CGN begin to die at 4-5 DIV (Blancas and Moran, 2011) and we do not observe a XIAP reduction at 2-3 DIV when there is not a decrease in the survival rate ~~diminish~~. During this time XIAP is able to bind and bring down the proapoptotic activity of caspase-3, that is well known to be one of the actions for its antiapoptotic function (for review, see Gyrd-Hansen and Meier, 2010; Mace et al., 2010). At 7 DIV the reduction of CGN viability correlates ~~is together~~ with a decrease in XIAP levels ~~decreased~~ and an increase in caspase-3 activity ~~is greater~~. These results could suggest that XIAP is related to neuronal survival promoted by depolarizant conditions.

In this regard, in CGN cultured in K5 and treated for 12 h with NMDA or K25 at 2 DIV, there are an increase of XIAP levels and its interaction with caspase-3 together with a reduction ~~reduce~~ of mitochondrial Smac release (Xifro et al, 2006). In this work, we determined that the interaction between XIAP and subunits of active ~~tetramer of~~ caspase-3 tetramer is maintained up to 6 DIV as well as neuronal viability does. This interaction is present in CGN under survival (K25 and K25-4d) or death (K5) conditions. However, in survival conditions the high levels of XIAP are enough to counteract the existent levels of active caspase-3 while in death conditions the low levels of XIAP ~~low levels~~ are not sufficient to neutralize all active caspase-3 and the caspase is free to carry out the death program. Similarly, in the cerebellum there is caspase-

3/XIAP interaction at P12 and P16 but only at P12, when occurs the massive death process of CGN (Koppel et al., 1983), there is active caspase-3 free. Moreover, the XIAP inhibition in CGN cultured under survival conditions induces an increase of apoptotic cells and a decrease of cell viability, consistent with the idea that XIAP has an important role in neuronal survival.

In summary, we suggest that the CGN survival under *in vitro* and *in vivo* conditions could be positively mediated by IAPs. Particularly, XIAP could has a critical role in CGN survival promoted by trophic effect of depolarization and in long-lasting survival of mature CGN; via its ability as caspases inhibitor and yet the cellular death program.

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Figure Legends

Figure 1: Time course of IAPs levels during cerebellar development. cIAP-1 (A), cIAP-2 (B), XIAP (C) and survivin (D) levels at different postnatal days (0, 4, 8, 12, 16 and 25) were measured by Western blot as detailed in Experimental Procedures. Above: representative immunoblot of IAP level. GAPDH was used as loading control; below: densitometric analysis of western blots. Data are expressed as the percent of IAP levels at P0. Values are mean $\pm$ S.E.M of eight (A), five (B), eight (C) and six (D) independent experiments. *, + and # indicate statistical significance *versus* P0, P4 or P8, respectively ($p<0.05$). ‡ indicates statistical significance *versus* P0-P16 ($p<0.05$).

Figure 2: Localization of IAPs on cerebellar cortex during development. The distribution of cIAP-2, survivin (Surv.) and XIAP on cerebellar sagittal sections at different postnatal days (4, 8, and 16) was measured by immunohistochemistry assays and nuclei were stained with DAPI as detailed in Experimental Procedures. Confocal images of developing cerebellum are shown; nuclei (DAPI) are shown in red and IAPs in green. The representative images of three independent experiments are oriented with external granule layer to left side. Scale bar corresponds to 50 μm.

Figure 3: cIAP-1, cIAP-2 and survivin levels of CGN on culture. mRNA levels of *cIAP-1*, *cIAP-2* and *survivin* (A) of CGN cultured in K25 or K5 at different DIV, were measured by RT-PCR as detailed in Experimental Procedures. *18s* mRNA levels were used as loading control of expression. cIAP-1 (B), cIAP-2 (C) and survivin (D) levels of CGN cultured in K25 or K5 at different DIV, were

measured by Western blot as detailed in Experimental Procedures. Above: representative immunoblot of IAP level. GAPDH was used as loading control. At 3 and 7 DIV some cells grown in K5 were also treated with K25 during the last day (K25-1d) or the last 5 days (K25-5d). Below: densitometric analysis of western blots. Results are expressed as the ratio IAP/GAPDH considering K5 at 2 DIV as 100%. Values are mean ± S.E.M of five (B), six (C) and four (D) independent experiments. * and # indicate statistical significance *versus* K5 at 2 DIV and K5 at 2 DIV, respectively ($p<0.05$).

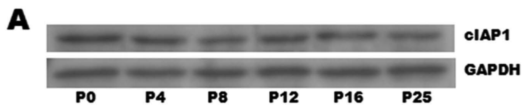
Figure 4: XIAP levels and neuronal survival of CGN on culture. mRNA levels of *xiap* (A) of CGN cultured in K25 or K5 at different DIV, were measured by RT-PCR as detailed in Experimental Procedures. *18s* mRNA levels were used as loading control of expression. XIAP levels (B) of CGN cultured in K25 or K5 at different DIV, were measured by Western blot as detailed in Experimental Procedures. Above: representative immunoblot of XIAP level. GAPDH was used as loading control. At 3 and 7 DIV some cells grown in K5 were also treated with K25 during the last day (K25-1d) or the last 5 days (K25-5d). Below: densitometric analysis of western blots. Results are expressed as the ratio XIAP/GAPDH considering K5 at 2 DIV as 100%. Values are mean ± S.E.M of six independent experiments. * and # indicate statistical significance *versus* K5 at 2 DIV and K5 at 2 DIV, respectively ($p<0.05$). The viability at 7 DIV (C) of CGN grown with K25, K5 and cells grown in K5 and treated with K25 during the last 5 days (K25-5d) was evaluated with MTT assays as mentioned in Experimental Procedures. Above: representative images of phase contrast of CGN cultured are shown; scale bar corresponds to 30 μm. Below: cell viability is

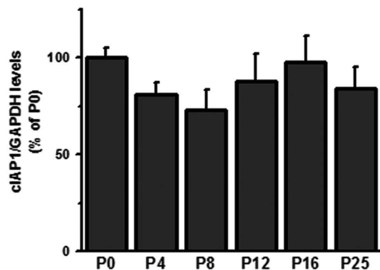
expressed as the percent of K25 (100%). Values are mean $\pm$ S.E.M of four independent experiments. Asterisks indicate statistical significance *versus* K25 ($p<0.05$).

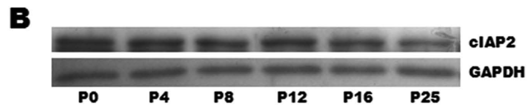
Figure 5: XIAP/Caspase-3 interaction of CGN on culture and in the cerebellum during development. The interaction of XIAP with active caspase-3 was measured by immunoprecipitation and Western blot as detailed in Experimental Procedures. The XIAP/caspase-3 interaction of CGN grown with K25, K5 and cells grown in K5 and treated with K25 during the last 4 days (K25-4d) was determinate at 6 DIV (A). In developing cerebellum, the XIAP/caspase-3 interaction was measured at P12 and P16 (B). In each panel, above: representative immunoblot of caspase-3 active subunit (p12), XIAP and IgG present in the immune complex recuperated. Middle: representative immunoblot of caspase-3 active subunit (p12) and XIAP present in the supernatant after removed the immune complex. Below: representative immunoblot of caspase-3 active subunit (p12) and XIAP present in the homogenates before immunoprecipitation. Representative images are of three independent experiments.

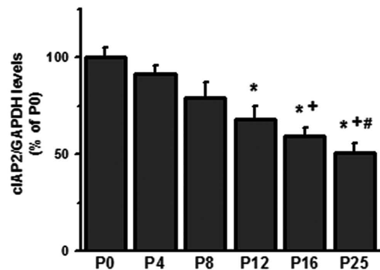
Figure 6: Inhibition of XIAP levels of CGN on culture. Cerebellar granule cells were infected when seeded with lentiviral shXIAP RNAi ($1x10^6$ TU/ml) or with a shscrambled sequence used as a control. *A*, Decreased levels of *xiap* mRNA verified the efficiency of XIAP down-regulation in granule neurons. *18s* mRNA levels were used as control of expression. *B*, XIAP protein levels were determined at 7DIV by western blotting. GAPDH protein levels were used as

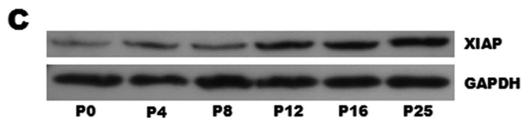
loading control. *C*, Representative photographs of infected neurons at 7DIV are shown. *D*, The percentage of apoptotic nucleus stained with Hoechst were determined at 7DIV with sub-toxic titration ($1x10^6$ TU/ml) and in 2 independent experiments performed in triplicate. *E*, The viability was measured by MTT reduction assay at 7DIV ($1x10^6$ TU/ml) in 3 independent experiments performed in triplicate. ** $p<0.01$ vs sh scrambled

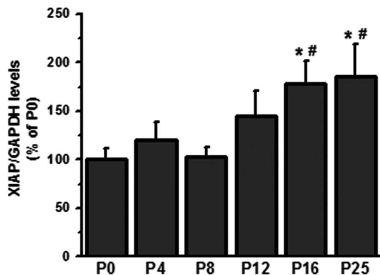

A
cIAP1
GAPDH
P0
P4
P8
P12
P16
P25

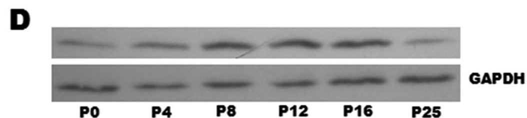

cIAP1/GAPDH levels
(% of P0)
0
50
100
P0
P4
P8
P12
P16
P25

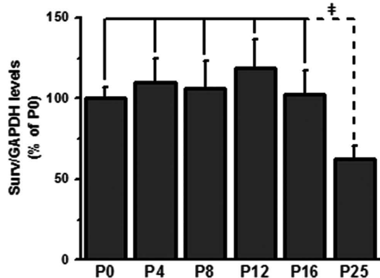

B
cIAP2
GAPDH
P0
P4
P8
P12
P16
P25


cIAP2/GAPDH levels
(% of P0)
0
50
100
*
*+
*+#
P0
P4
P8
P12
P16
P25


C
XIAP
GAPDH
P0
P4
P8
P12
P16
P25

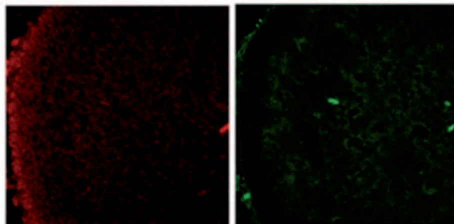

XIAP/GAPDH levels
(% of P0)
0
50
100
150
200
250
*#
*#
P0
P4
P8
P12
P16
P25

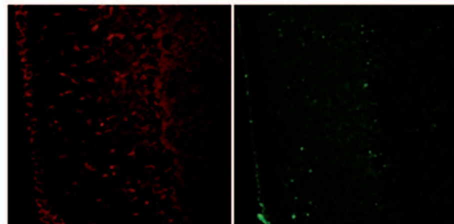

D
GAPDH
P0
P4
P8
P12
P16
P25


Surv/GAPDH levels
(% of P0)
0
50
100
150
‡
P0
P4
P8
P12
P16
P25

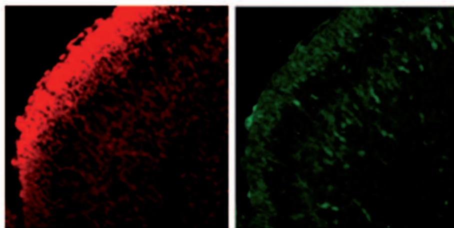
P4 P16

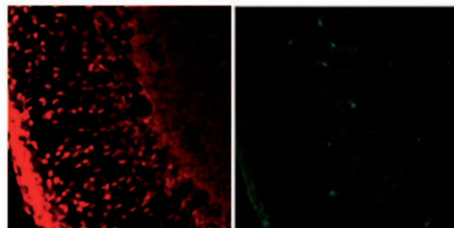
cIAP2





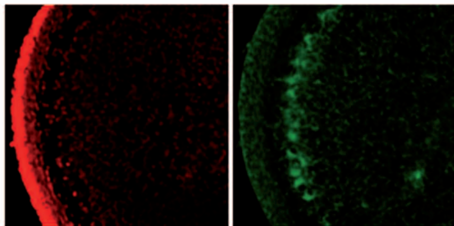
Surv.

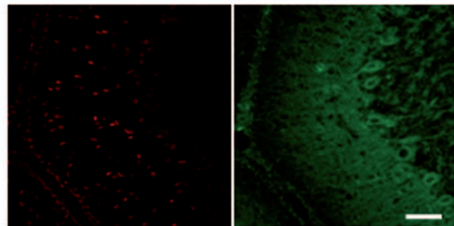


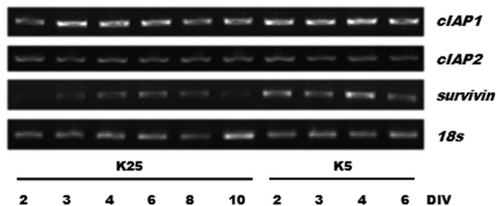


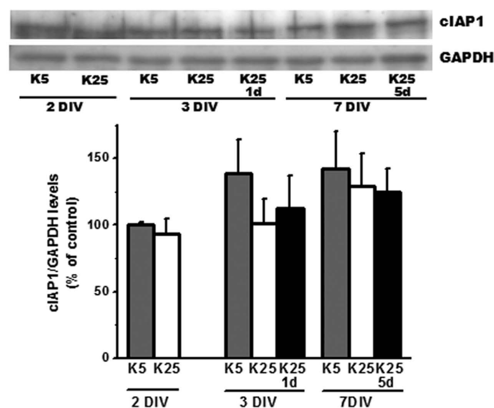
P8 P16

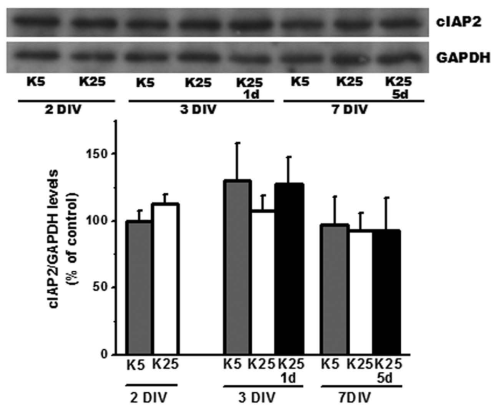
XIAP

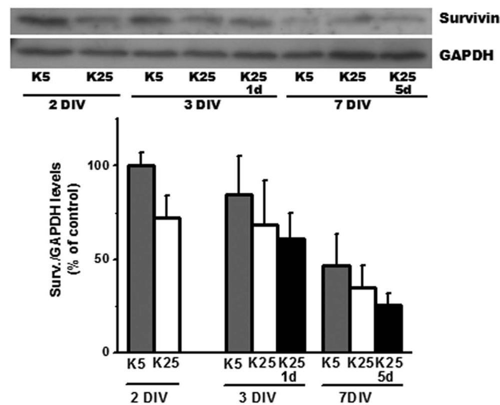




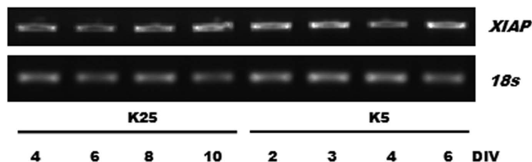

A
cIAP1
cIAP2
survivin
18s
K25
K5
2 3 4 6 8 10 2 3 4 6 DIV


B
cIAP1
GAPDH
K5 K25 K5 K25 K25 1d K5 K25 K25 5d
2 DIV
3 DIV
7 DIV
cIAP1/GAPDH levels (% of control)
150
100
50
0
K5 K25 K5 K25 K25 1d K5 K25 K25 5d
2 DIV
3 DIV
7DIV

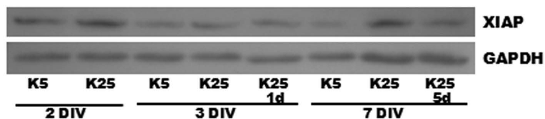

C
cIAP2
GAPDH
K5 K25 K5 K25 K25 1d K5 K25 K25 5d
2 DIV
3 DIV
7 DIV
cIAP2/GAPDH levels (% of control)
150
100
50
0
K5 K25 K5 K25 K25 1d K5 K25 K25 5d
2 DIV
3 DIV
7DIV

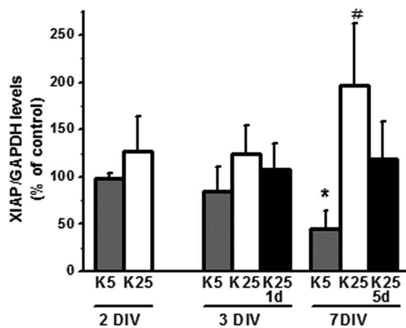

D
Survivin
GAPDH
K5 K25 K5 K25 K25 1d K5 K25 K25 5d
2 DIV
3 DIV
7 DIV
Surv./GAPDH levels (% of control)
100
50
0
K5 K25 K5 K25 K25 1d K5 K25 K25 5d
2 DIV
3 DIV
7DIV

A



B





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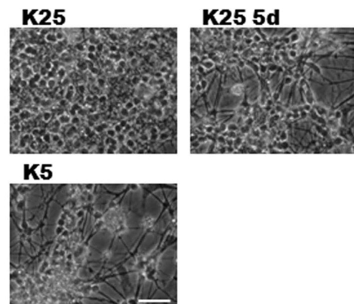
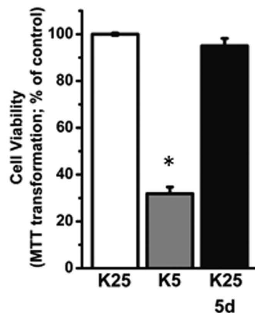




A
IP: XIAP

Casp-3
p12

XIAP

IgG
Supernatant IP

Casp-3
p12

XIAP
Total homogenate

Casp-3
p12

XIAP
K25
K5
K25 4d
B
IP: XIAP

Casp-3
p12

XIAP

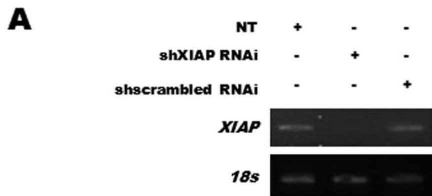
IgG
Supernatant IP

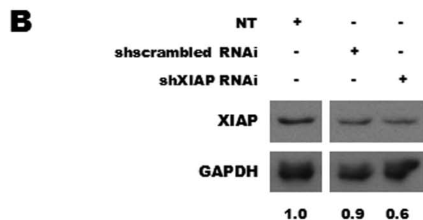
Casp-3
p12

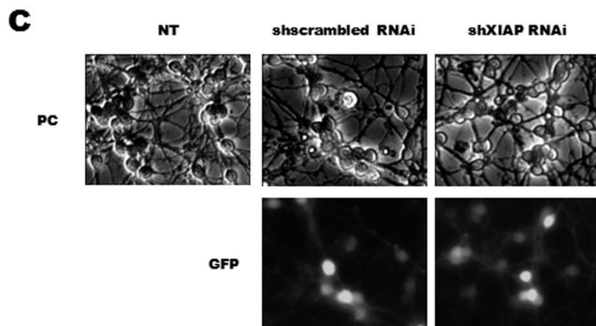
XIAP
Total homogenate

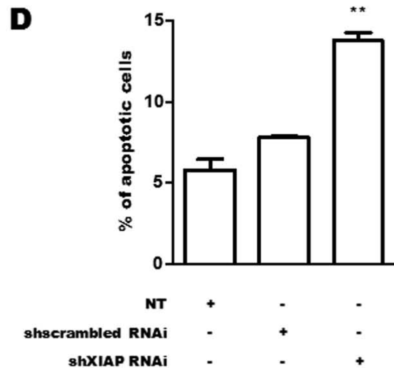
Casp-3
p12

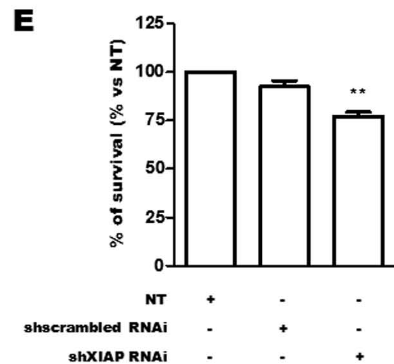
XIAP
P12
P16


A
NT
shXIAP RNAi
shscrambled RNAi
XIAP
18s


B
NT
shscrambled RNAi
shXIAP RNAi
XIAP
GAPDH
1.0
0.9
0.6


C
NT
shscrambled RNAi
shXIAP RNAi
PC
GFP


D
% of apoptotic cells
NT
shscrambled RNAi
shXIAP RNAi


E
% of survival (% vs NT)
NT
shscrambled RNAi
shXIAP RNAi