

Quinto trabajo

“Bax deficiency promotes a differential up-regulation of the BH3-only protein Bim_{EL} and Bak during striatal and cortical development, and after excitotoxic injury”

En preparación

Bax deficiency promotes a differential up-regulation of the BH3-only protein Bim_{EL} and Bak during striatal and cortical development, and after excitotoxic injury

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ABSTRACT

Bax, the pro-apoptotic member of the Bcl-2 family, is a key regulator of programmed cell death during development and also participates in the apoptotic processes in some injury models. However, targeted disruption of this protein produces limited phenotypic abnormalities during development, but excitotoxic cell death can be partially prevented in adult striatum. Here, we have examined the compensatory mechanisms activated by the lack of Bax during striatal and cortical postnatal development, and in striatal excitotoxic lesion. Although morphological analysis showed the same number of cortical and striatal neurons in adult Bax deficient mice compared to wild-type animals, pro-apoptotic proteins, Bim, Bak and Bad were differentially regulated in Bax null mutant animals. Bim protein levels increased in both brain areas examined but with a distinct temporal pattern. On the other hand, Bak was only increased at early stages of cortical postnatal development, whereas Bad was unchanged. Furthermore, pro-survival proteins Bcl-2 and Bcl-x_L were not modified by the lack of Bax at any postnatal ages analyzed. Excitotoxic injury induced in the striatum differentially modified these proteins in wild-type and Bax knockout animals. Bim protein levels were up-regulated in both genotypes, whereas a specific enhancement of Bak and Bcl-x_L were only observed in Bax null mutant mice after quinolinate lesion. In conclusion, our results show that in the absence of Bax, other members of the Bcl-2 family, such as Bim and Bak, are specifically regulated suggesting that these proteins could compensate Bax function during postnatal development as well as in response to excitotoxicity.

INTRODUCTION

Apoptotic cell death is crucial in the normal development of the nervous system. However, this process also takes place in adult organisms in response to acute or chronic insults underlying several neurodegenerative disorders (Vila and Przedborski, 2003; Ekshyyan and Aw, 2004). Apoptosis can be activated through the extrinsic and intrinsic cell death pathways (Hengartner, 2000; Danial and Korsmeyer, 2004). Death signals activating the intrinsic pathway affect mitochondrial function leading to the release of apoptosis-regulatory factors from mitochondria that participate in the initiation of the caspase cascade in the cytosol (Hengartner, 2000; Danial and Korsmeyer, 2004). One of the major regulators of the mitochondrial integrity is the Bcl-2 family of proteins (Green and Kroemer, 2004; Lucken-Ardjomande and Martinou, 2005). This family comprises both pro-apoptotic and anti-apoptotic proteins. Pro-apoptotic proteins cause mitochondrial dysfunction leading to the release of apoptogenic factors whereas anti-apoptotic members of the Bcl-2 family prevent those events (Cory and Adams, 2002).

Proteins belonging to the Bcl-2 family share, at least, one conserved region termed Bcl-2 homology (BH) that mediate protein-protein interactions. Anti-apoptotic members, such as Bcl-x_L and Bcl-2, contain four BH domains whereas the pro-apoptotic members of this family are divided into two subgroups: proteins that contain multiple BH domains, such as Bax and Bak; and BH3-only members, such as Bad and Bim (Puthalakath and Strasser, 2002). Interactions and the intracellular balance between members of the Bcl-2 family regulate whether a cell lives or dies after death stimuli. Thus, for example, Bcl-2 and Bcl-x_L can inhibit Bax through its heterodimerization (Merry and Korsmeyer, 1997), meanwhile Bim can suppress Bcl-2 and Bcl-x_L pro-survival properties sequestering them from Bax after being released from the dynein light chain LC8 (Puthalakath et al., 1999). Furthermore, the function of Bcl-2 family members can also be modified in a transcriptional manner (Puthalakath and Strasser, 2002; Cory and Adams, 2002). In fact, increased expression of Bax (Gillardon et al., 1995; Krajewski et al., 1995; Vis et al., 2001; Martin et al., 2003; Krasnova et al., 2005; Perez-Navarro et al., 2005) or Bim (Napankangas et al., 2003; Okuno et al., 2004; Shinoda et al., 2004; Gao et al., 2005) and reduced levels of Bcl-2 and/or Bcl-x_L

(Gillardon et al., 1995; Krajewski et al., 1995; Krasnova et al., 2005; Perez-Navarro et al., 2005) have been detected in response to several injuries to the nervous system.

In accordance to the role of Bcl-2 family members in the apoptotic processes after insults to the nervous system, Bax knockout (KO) mice are less sensitive to various cell death stimuli. Bax KO cultured sympathetic neurons show resistance to trophic factor deprivation (Deckwerth et al., 1996; Putcha et al., 2000) as well as cerebellar neurons to potassium deprivation (Miller et al., 1997) and motoneurons to trophic factor or serum withdrawal (Deckwerth et al., 1996; Bar-Peled et al., 1999). Similarly, Bax deficiency attenuates kainate-induced apoptosis in cultured motoneurons (Bar-Peled et al., 1999) and apoptosis of cultured cortical cells after exposure to glutamate (Xiang et al., 1998). Furthermore, Bax KO mice show less dopaminergic cell death after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine administration (Vila et al., 2001), resistance to ischemia-mediating neuronal loss in the hippocampus (Gibson et al., 2001) and a reduction in striatal cell death induced by quinolinate (QUIN) or kainate injection (Perez-Navarro et al., 2005).

Although all these studies show the involvement of Bax in stimulus-induced cell death, the role of Bax in naturally occurring cell death seems specific to some neuron populations and the developmental stage (Deckwerth et al., 1996; Shindler et al., 1997; White et al., 1998; Fan et al., 2001) suggesting that other Bcl-2 family members could participate in this process. Therefore, the aim of the current study is to determine which mechanisms are triggered by Bax deficiency on members of the Bcl-2 family during postnatal development and after an excitotoxic lesion.

MATERIALS AND METHODS

Materials

Heterozygous Bax mice were purchased from Jackson Laboratories (Bar Harbor ME, USA). QUIN and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Bax primers were from Qiagen (Hilden, Germany). Apoptosis detection system (fluorescein) and horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were obtained from Promega (Madison, WI, USA).

For Western blot analysis Bax and Bad antibodies were from Cell Signaling (Beverly, MA), Bcl-x_L and Bcl-2 antibodies were from Transduction Laboratories (Lexington, KY, USA), Bim antibody was from Stressgen (San Diego, CA, USA), Bak antibody was from Upstate (Charlottesville, VA, USA), α -tubulin antibody was from Sigma Chemical Co. (St. Louis, MO, USA), and polyvinylidene difluoride membranes (Immobilon-P) were from Millipore (Massachusetts, MA, USA). Enhanced chemiluminescence (ECL) was purchased from Amersham Biosciences (Freiburg, Germany). For immunohistochemistry, NeuN antibody was purchased from Chemicon (Temecula, CA, USA), and the Avidin-Biotin Complex (ABC) kit was from Pierce (Tattenhall, UK). Phoretix 1D Gel analysis software was from Phoretix International Ltd. (Newcastle, UK).

Animal subjects

Heterozygous Bax (C57BL/6J) mice were bred to maintain the colony and to obtain Bax ^{-/-}, ^{+/-}, and wild-type genotypes. Genotyping was performed as described elsewhere (Perez-Navarro et al., 2005).

Male wild-type and Bax KO mice were sacrificed at different postnatal days (P; 3, 7, 15 and 21) and in the adulthood (8 weeks) to perform Western blot analysis (n = 5 for each genotype and time point analyzed). Animal treatments and handling procedures were approved by the Local Committee (99/1 University of Barcelona) and the Generalitat de Catalunya (1094/99), in accordance with the European Communities Council Directive (86/609/EU).

Excitotoxic lesion

Wild-type and Bax KO mice were anesthetized with pentobarbital and QUIN (24 nmol) was intrastrially injected at the following coordinates relative to bregma: AP +0.6 mm, ML +2 mm and 2.7 mm below the dural surface with the incisor bar at 3 mm above the interaural line. After surgery, animals were housed separately with food and water ad libitum in a colony room maintained at a constant temperature (19-22°C) and humidity (40-50 %) on a 12:12 hr light/dark cycle.

For Western blot analysis animals were killed by decapitation (n = 5 for each genotype and time point) at 24 and 48 h post-lesion. Brains were rapidly removed and

striata ipsilateral as well as contralateral to the lesion were dissected and frozen at -80°C. For the in situ detection of DNA fragmentation mice at 24, 48 and 72 h post-injection were deeply anaesthetized and immediately perfused transcardially with PBS followed by 4% paraformaldehyde/phosphate buffer (0.1 M, pH 7.4) (n = 4 for each genotype and time point). Brains were rapidly removed and postfixed for 1-2 h in the same solution and cryoprotected by immersion in 15 % sucrose/PBS.

Western blot analysis.

To examine striatal and cortical protein levels of Bax, Bak, Bcl-2, Bcl-x_L, Bim and Bad Western blot was performed as described elsewhere (Perez-Navarro et al., 2005). Antibodies used were: Bcl-2 (mouse monoclonal, 1:2000), Bcl-x_L (mouse monoclonal, 1:500), Bak (rabbit polyclonal, 1:2000), Bim (rabbit polyclonal, 1:1000), Bad (rabbit polyclonal, 1:1000) or Bax (rabbit polyclonal, 1:2000). As loading controls membranes were re-incubated with a monoclonal anti- α -tubulin antibody (1:50,000). After two rinses with TBS-T (Tris buffer saline-tween), membranes were incubated for 1 h with anti-rabbit or anti-mouse Ig linked secondary antibodies (1:2000), and the reaction was finally visualized using a chemiluminescence detection system (ECL). Western blot replicates were scanned and quantified using the Phoretix 1D Gel Analysis.

In situ detection of DNA fragmentation

Brains were frozen in dry ice-cooled isopentane and cryostat horizontal sections (14 μ m) through the whole striatum were serially collected on silane-coated slides. DNA fragmentation was histologically examined using the in situ Apoptosis detection system, Fluorescein and performed as described elsewhere (Perez-Navarro et al., 2000).

Immunohistochemistry

For immunohistochemical analysis, adult animals (n = 5 for each condition) were deeply anesthetized and immediately perfused through the heart with saline followed by 4 % paraformaldehyde/phosphate buffer. Brains were removed and postfixed for 1-2 h in the same solution, cryoprotected by immersion in 30 % sucrose/PBS and then frozen in dry ice-cooled isopentane. Cryostat serial horizontal sections (40 μ m) through the whole striatum were serially collected in PBS as free-floating sections and stained with the NeuN (1:100) antibody. After treatment with H₂O₂ (0.3 % in PBS, 10 % methanol)

for 15 min and blocking with 5 % normal horse serum and 0.2 % BSA for 2 h, sections were incubated with primary antibody for 16 h at 4 °C. After washing, they were incubated with biotinylated secondary antibodies (1:200) and then with the avidin-biotin complex (ABC kit). Finally, sections were developed with 0.05 % diaminobenzidine, 0.01 % NiCl₂ and 0.02 % H₂O₂. As negative immunohistochemical controls, some sections were processed as described above in the absence of primary antibody.

Cell counting

Stereological quantitation was carried out using an optical dissector/Cavalieri combination as previously described by Oorschot (1996). All cell counts were performed blind with respect to genotype. Stereological counts were obtained from the entire neostriatum and from 300 μm² of layer V of motor cortex using the Computer Assisted Stereology Toolbox (CAST) software (Olympus Denmark A/S, Ballerup, Denmark). The dissector counting method was employed to analyse every seventh coronal sections of the brain area containing the striatum or cortex. The counting frames were randomly sampled. The coefficient of variation was between 0.05-0.08.

RESULTS

Bax KO mice do not show striatal or cortical abnormalities

To test whether Bax deficiency could affect the development of the striatum and cortex, the number of NeuN-positive cells in these regions was examined in adult animals. No significant differences were observed in the density of NeuN-positive cells (cells /mm²) between wild-type and Bax KO animals, in any of the regions examined (Striatum: wild-type, 83,340 ± 7877; KO, 86,280 ± 5240; Cortex: wild-type, 53,902 ± 7177; KO, 48,697 ± 1909). Furthermore, striatal volume (wild-type: 10.8 ± 0.2 mm³; KO: 11.1 ± 0.2 mm³) and the total striatal cell number (wild-type: 896,876 ± 81,825; KO: 961,155 ± 75,500) was similar in both genotypes.

To examine the possible contribution of Bax to the development of the striatum and cortex, its protein levels were quantified in wild-type animals at different postnatal ages. Bax showed a similar time course in both brain areas, with maximum levels at P3

and P7 (Fig. 1). At P15 protein levels decreased until the adulthood (Fig. 1) with a higher decrease in the cortex (Fig. 1A).

Pro-apoptotic, but not pro-survival, protein levels are differentially increased in Bax KO mice

Wild-type and Bax KO adult mice had similar striatal and cortical cell density suggesting that other Bcl-2 family members could compensate Bax deficiency. Thus, we examined protein levels of Bak, Bim, Bad, Bcl-2 and Bcl-x_L during the postnatal development of wild-type and Bax KO mice.

In contrast to Bax, Bak protein followed a different pattern of expression in the cortex and striatum during postnatal development. In the cortex of wild-type animals it showed two peaks of expression, at P7 and P21 (Fig. 2A) while in the striatum Bak levels were similar from P3 to P21 with a dramatic decrease in the adulthood (Fig 2B). In Bax KO mice Bak protein levels in cortex, but not in the striatum, were modified compared to wild-type animals (Fig. 2). We observed an increase (by 67%), but only at P3 (Fig. 2A).

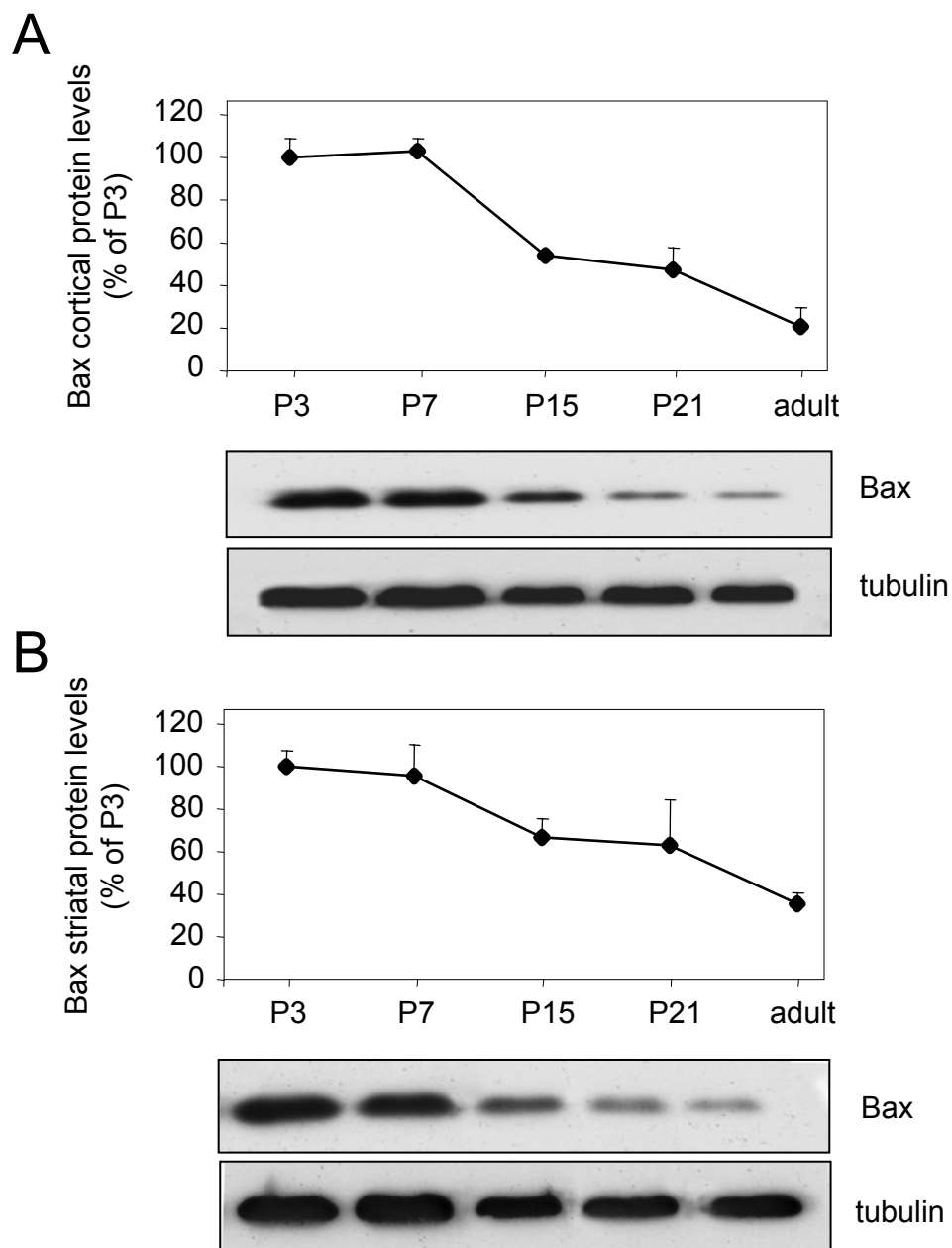


Figure 1.- Bax follows a similar pattern of expression during cortical and striatal postnatal development. The expression levels of Bax were examined by Western blot in the cortex (A) and striatum (B) at different postnatal ages. Results obtained from densitometric measures are expressed as percentages of P3 values \pm SEM for five animals per condition. Immunoblots were obtained from representative experiments.

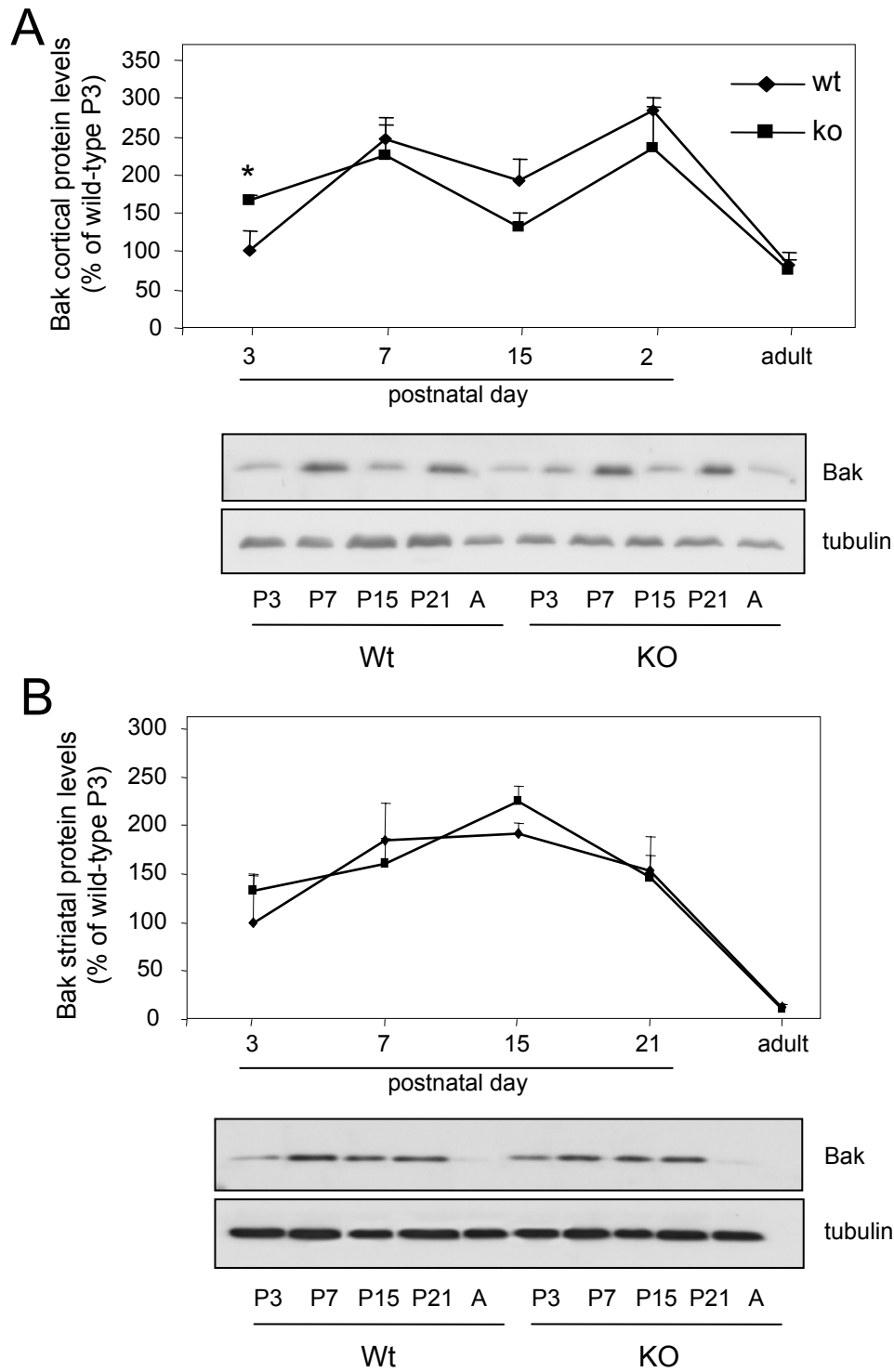


Figure 2.- Bak is differentially expressed, and regulated in the absence of Bax, in cortex and striatum during postnatal development. Cortical (A) and striatal (B) Bak protein levels were analyzed by Western blot in wild-type (diamond; Wt) and Bax KO (square; KO) mice at different postnatal ages. Results obtained from the analysis of western blots are expressed as a percentage of P3 wild-type values \pm SEM for five animals per condition. In Bax KO animals, increased levels of Bak were only observed in the cortex at P3. * $p < 0.05$, compared with P3 values in wild-type animals. Statistical analysis was performed by Student's *t*-test. Immunoblots show representative experiments.

Bim expression in wild-type animals also showed a different pattern during cortical and striatal postnatal development (Fig. 3). In the cortex, its levels of expression were similar between P3 until the adulthood (Fig. 3A) whereas in the striatum, the maximum levels were detected at P3 decreasing until P15 and maintained until the adulthood (Fig. 3B). When compared to wild-type animals, Bax KO mice showed increased Bim protein levels in both brain areas but at different postnatal ages (Fig. 3). In the cortex, up-regulated levels were detected at all the ages examined although the increase was higher at P3 and P7 (Fig. 3A). In contrast, striatal Bim protein levels were only modified at later postnatal ages (P21 and adulthood; Fig. 3B).

When the expression of Bad was analyzed, differences between cortex and striatum were also observed (Fig. 4). In wild-type animals, cortical Bad protein levels progressively increased over the time reaching its maximum in the adulthood (Fig. 4A). In contrast, striatal Bad protein expression slightly increased at P7 with maximum levels detected at P21 (Fig. 4B). No differences between wild-type and Bax KO animals were observed in the expression pattern of Bad during postnatal development in any of the brain regions examined (Fig. 4).

Finally, pro-survival proteins were also examined. In wild-type animals, the expression pattern of Bcl-2 was similar in both brain areas showing maximum levels at P3-P7 and a decrease at P15 that was maintained until the adulthood (Fig. 5). In contrast, Bcl-x_L was differentially expressed in the cortex and striatum during postnatal development. Cortical Bcl-x_L protein levels peaked at P21 (Fig. 5C) whereas in the striatum maximum levels were reached at P7 (Fig. 5D). In Bax KO mice, the pattern of expression of Bcl-2 and Bcl-x_L in the cortex and striatum was similar to wild-type animals (Fig. 5C and 5D).

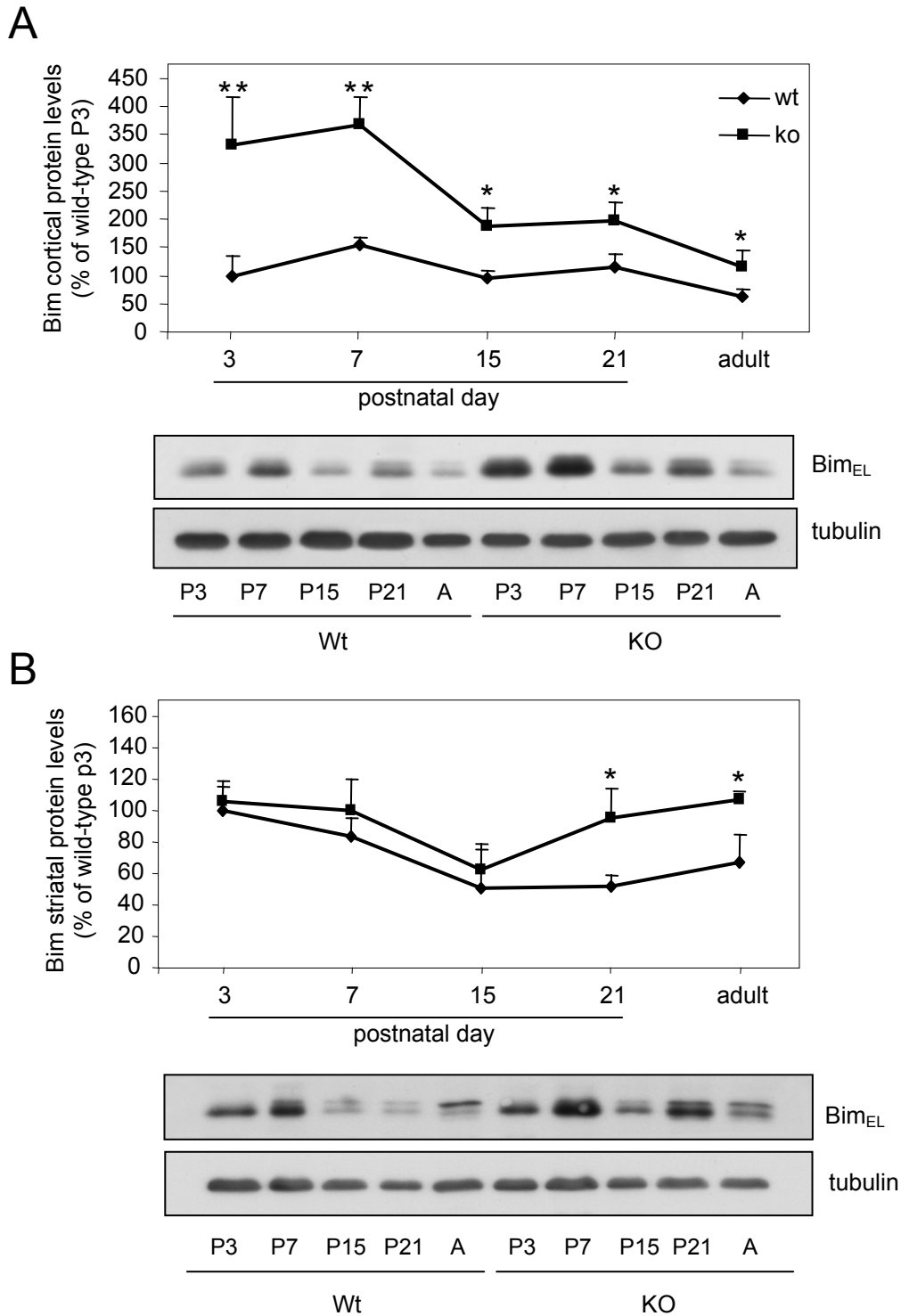


Figure 3.- Bax deficiency increases Bim protein levels in the cortex and striatum following a different temporal pattern. Cortical (A) and striatal (B) protein levels of Bim were analyzed by Western blot at different postnatal ages in wild-type (diamond, Wt) and Bax KO (squares, KO) mice. Results are expressed as a percentage of protein levels in P3 wild-type mice \pm SEM for five animals per condition. Increased levels of Bim protein were detected in the cortex of Bax KO mice at all the postnatal ages analyzed whereas in the striatum the increase was restricted to P21 and adult mice. ** $p < 0.01$, * $p < 0.05$ compared with corresponding values in wild-type animals. Statistical analysis was performed by Student's *t*-test. Immunoblots were obtained from representative experiments.

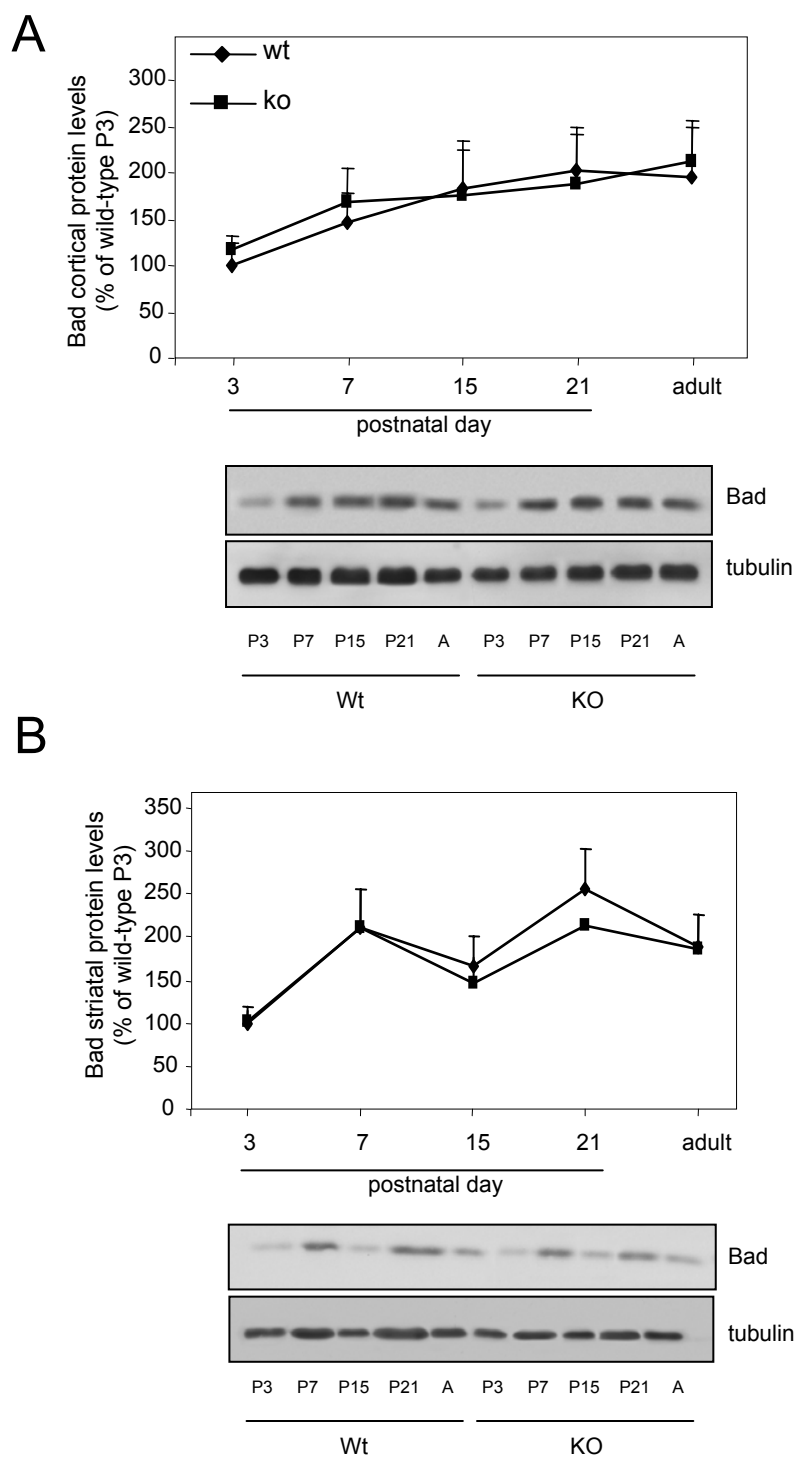


Figure 4.- Similar expression of Bad protein in wild-type and Bax KO mice. Bad protein was analyzed by immunoblotting during postnatal development in cortex (A) and striatum (B) of wild-type (diamond; Wt) and Bax KO (square; KO) mice. Results obtained from densitometric measures are expressed as a percentage of P3 wild-type values \pm SEM for five animals per condition. No differences were observed between wild-type and Bax KO animals. Immunoblots show representative experiments.

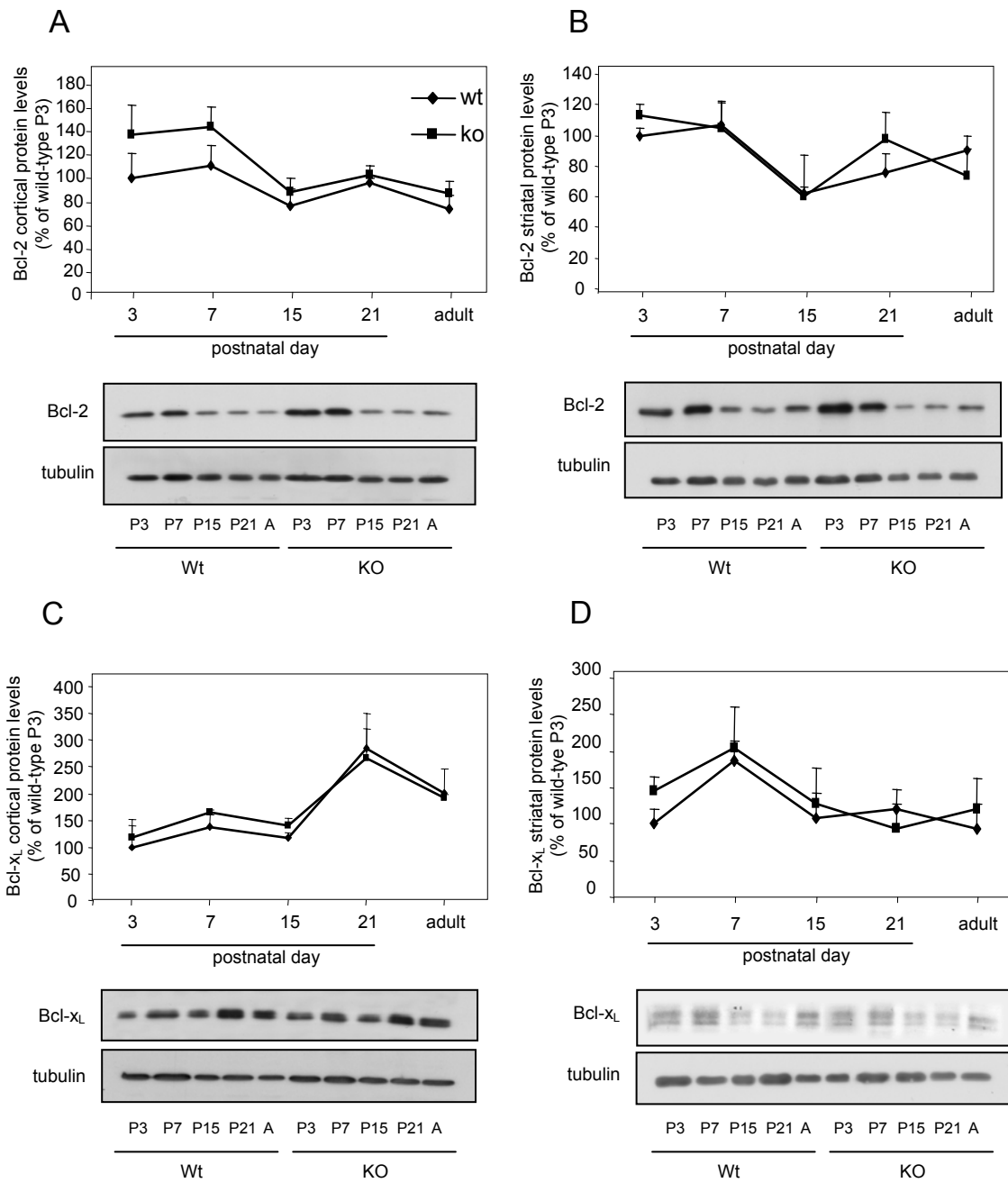


Figure 5.- Bax deficiency does not affect Bcl-2 and Bcl-x_L protein expression. Protein levels of Bcl-2 (A and B) and Bcl-x_L (C and D) were analyzed by Western blot at different postnatal ages in the cortex (A and C) and striatum (B and D) of wild-type (diamonds, Wt) and Bax KO mice (squares, KO). Results are expressed as a percentage of protein levels in wild-type P3 ± SEM for five animals per condition. Immunoblots were obtained from representative experiments.

Excitotoxicity differentially regulates Bcl-2 family members in wild-type and Bax KO animals

In order to examine the regulation of Bcl-2 family members by excitotoxicity, QUIN was injected in the striatum of wild-type and Bax KO mice. First, we analyzed cell death at 24, 48 and 72 h after lesion by the TUNEL assay. Positive nuclei were detected at 24 h although they increased at 48 and 72 h post-lesion (data not shown).

The analysis of Bim protein levels disclosed an increase in both wild-type (by $80 \pm 32\%$) and Bax KO (by $47 \pm 11\%$) animals, 48 h after intrastriatal QUIN injection (Fig. 6A). In contrast, protein levels of Bak (by $105 \pm 25\%$) and Bcl-x_L (by $36 \pm 10\%$) were only up-regulated in Bax KO animals (Fig. 6B and 6C, respectively). Moreover, neither Bcl-2 (wild-type: $92 \pm 4\%$, Bax KO: $106 \pm 9\%$; % of contralateral side) nor Bad (wild-type: $86 \pm 6\%$, Bax KO: $93 \pm 9\%$; % of contralateral side) protein levels were modified by intrastriatal QUIN injection.

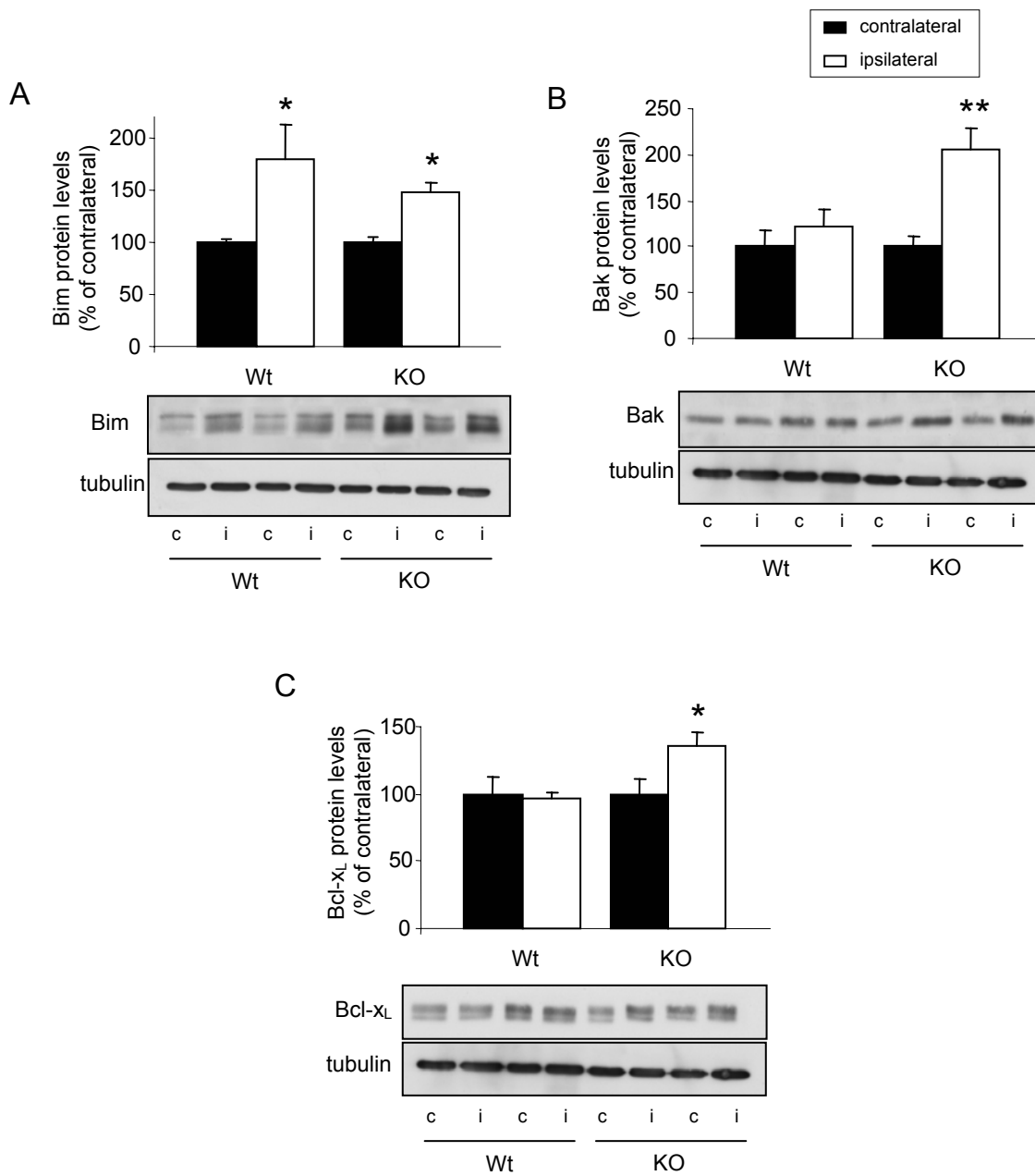


Figure 6.- Bcl-2 family members are differentially regulated in wild-type and Bax KO animals by the intrastriatal injection of QUIN. Bim (A), Bak (B) and Bcl-x_L (C) were analyzed by immunoblotting 48 h after QUIN injection in wild-type (Wt) and Bax KO striata (KO). Results obtained from the Western blot quantification are expressed as a percentage of protein levels in the non-lesioned (contralateral) striata ± SEM for five animals per condition. Bim protein levels (A) were similarly increased by intrastriatal QUIN injection in wild-type and Bax KO animals whereas the levels of Bak (B) and Bcl-x_L (C) were only increased in Bax KO mice. ***p* < 0.01, **p* < 0.05 compared with the contralateral striatum. Statistical analysis was performed by Student's *t*-test. c: contralateral striatum to the lesion (filled bars); i: ipsilateral striatum to the lesion (open bars).

DISCUSSION

Present data show that Bax protein levels are regulated during the development of striatum and cortex following a similar pattern of expression. However, the lack of Bax does not modify the normal development of these two brain areas. Here, we describe compensatory mechanisms that are triggered in the absence of Bax during postnatal development as well as after excitotoxicity. Bim expression is up-regulated in cortex and striatum, but at different postnatal ages, whilst Bak protein levels are only increased in the cortex of P3 animals. Furthermore, intrastriatal QUIN injection increases Bim protein levels in both genotypes examined whereas Bak and Bcl-x_L are only upregulated in Bax null mutant mice.

Our results show that the death promoting protein Bax is expressed in the cortex and striatum during postnatal development following a similar pattern of expression. The highest levels of Bax protein were detected at P3 and P7, suggesting an involvement of this protein in the cell death that takes place during these developmental stages (Fishell and Van der Kooy, 1991; Spreafico et al., 1995; Maciejewska et al., 1998). Similar results of Bax protein expression during rat cortical development have been previously reported (Vekrellis et al., 1997; Shimohama et al., 1998). Furthermore, our results are in agreement with the statement that Bax regulates the survival of postmitotic neurons (White et al., 1998). However, the stereological analysis of number of neurons in cortex and striatum in adult Bax KO mice did not show any differences compared to wild-type animals, suggesting the existence of alternative pathways to control apoptosis during cortical and striatal postnatal development. Furthermore, it is noteworthy that Bax effect might be cell-specific, since other selected populations of the peripheral and central nervous system are altered in Bax KO mice (White et al., 1998).

In contrast to that observed for Bax protein expression during postnatal development, other three pro-apoptotic proteins analyzed showed different pattern of expression in the cortex and striatum. Bak protein in the cortex showed two peaks of expression (at P7 and P21), whereas in the striatum its protein levels were maintained between P3-P21 and dramatically decreased in the adulthood. Similarly, a previous study shows that Bak protein levels in brain structures decreased during postnatal life (Krajewska et al., 2002). Regarding to Bim protein expression, its levels were constant in cortex during postnatal development. In contrast, Bim expression in striatum

decreased from P3 to reach adult levels at P15. It has been previously described that Bim is expressed at least partially in the same tissues as Bak or Bax (Krajewski et al., 1994, 1996; O'Reilly et al., 2000). Our results support these observations as all three proteins were detected in the cortex and striatum although with a different temporal pattern of expression. The other BH3-only protein analyzed, Bad, slightly increased its levels during cortical postnatal development, and in the striatum maximum levels were detected at late stages of postnatal development. It is interesting to point out that in contrast to Bax and Bak, BH-3 only proteins were not decreased in late postnatal stages. In fact, Bim expression was constant during cortical postnatal development, whereas Bad increases with age in both areas examined. These results could be related to the proposed role for BH3-only proteins as sensors of cell stress (Huang and Strasser, 2000).

The analysis of pro-survival proteins disclosed that Bcl-2 followed a similar expression pattern in cortex and striatum with a decrease at P15 that was maintained until the adulthood. In agreement, previous results also show that Bcl-2 protein levels in the central nervous system decrease with age (Ferrer et al., 1994; Merry et al., 1994; Shimohama et al., 1998; Mooney and Miller, 2000). In contrast to Bcl-2, Bcl-x_L maximum levels in cortex and striatum were detected at different postnatal ages although in both regions this protein was expressed in the adulthood at similar levels to P3. Other studies also described that Bcl-x_L is maintained at high levels postnatally in the central nervous system (Gonzalez-Garcia et al., 1995; Parsadanian et al., 1998; de Bilbao et al., 1999). Similarly, Bax was also detected at appreciable levels in adult cortex and striatum as previously described in mature neurons (Krajewski et al., 1994). Thus, this expression patterns raise the possibility that Bax and Bcl-x_L may be the most important regulators of apoptosis as neurons mature.

When Bcl-2 family protein expression was examined in the Bax KO mice no changes were observed in the pro-survival proteins whereas pro-apoptotic proteins were specifically regulated in the cortex and striatum. Bak protein levels were increased in the cortex but only at P3, Bim protein was up-regulated in cortex and striatum at different postnatal ages whereas Bad was not modified. Up-regulated levels of pro-apoptotic proteins in the absence of Bax are consistent with the hypothesis that the balance between anti- and pro-apoptotic Bcl-2 family members regulates the susceptibility of a cell to apoptosis (Oltvai et al., 1993; Shindler et al., 1997). The analysis of Bax protein levels during striatal and cortical development disclosed a

similar pattern of expression. Therefore, as Bax and Bak seem in most circumstances to be functionally equivalent (Lindsten et al., 2000; Wei et al., 2001) we expected a similar regulation of Bak protein in the striatum and cortex in the absence of Bax. However, Bak protein levels were only increased in the cortex at P3 suggesting that the relative importance of each member of the Bcl-2 family can vary in specific brain regions. The most striking feature observed was that Bax KO mice showed increased levels of Bim in both brain areas examined. Increased levels in the cortex were observed at all the postnatal ages examined, with maximum levels at P3-P7, with an expression pattern that parallels that of Bax in the cortex of wild-type animals. Since BH3-only proteins function upstream of Bax/Bak in apoptosis signaling (Cheng et al., 2001; Zong et al., 2001; Huang and Strasser, 2000) our results suggest that in Bax KO mice this protein could participate in the apoptotic processes that take place during cortical postnatal development, regulating Bak function. Furthermore, in the striatum Bim was only up-regulated at late postnatal ages pointing to a role in the control of apoptotic processes after cell death stimuli.

We have previously shown that Bax KO mice are partially resistant to QUIN-induced excitotoxicity in the striatum (Perez-Navarro et al., 2005). In the present work we examined whether other Bcl-2 family members could be regulated in this lesion paradigm in the absence of Bax. Bcl-2 and Bad did not show changes after intrastriatal QUIN injection in any of the genotypes analyzed. In contrast, excitotoxicity increased Bim protein levels in both genotypes according to previous studies showing up-regulation of Bim after several types of cell death stimuli *in vitro* (Harris and Johnson, 2001; Putcha et al., 2001; Whitfield et al., 2001; Gilley et al., 2003) and *in vivo* (Napankangas et al., 2003; Shinoda et al., 2004; Okuno et al., 2004; Gao et al., 2005). It is interesting to notice that although Bim protein levels were higher in the striatum of Bax KO mice its protein levels were similarly up-regulated in both genotypes. The fact that Bim acts upstream of Bax in neurons (Putcha et al., 2001) could explain this result. Therefore, our results suggest that Bim levels could set the threshold for initiation of apoptosis after an excitotoxic stimulus. Moreover, we also observed that in Bax KO mice, but not in wild-type animals, Bak expression was up-regulated after excitotoxicity. Studies in Bax/Bak double mutant mice disclose that these two pro-apoptotic proteins have overlapping roles in the regulation of apoptosis (Lindsten et al., 2000; Wei et al., 2001; Degenhardt et al., 2002) that could explain the increase in Bak protein levels in

the absence of Bax. Although these increased levels of Bak, cell death after QUIN injection in Bax KO animals is reduced compared to wild-type animals (Perez-Navarro et al., 2005). Interestingly, up-regulated levels of Bcl-x_L were also observed after intrastriatal QUIN injection in Bax deficient mice. Although Bcl-x_L can bind to Bax, its preferred heterodimerization partner appears to be Bak (Chittenden et al., 1995; Sattler et al., 1997). Furthermore, it has been previously described that Bak is sequestered by Bcl-x_L and Mcl-1, but not Bcl-2, until displaced by BH3-proteins (Willis et al., 2005). These results combined to the fact that Bim binds preferentially to Bcl-2 (Bouillet et al., 2001) allow us to speculate that increased levels of Bcl-x_L can be sequestering Bak and preventing apoptosis in Bax KO striatum. However, it has been recently described that Bax deficiency prevents Bak oligodimerization and prevents cytochrome *c* release (Mikhailov et al., 2003). Therefore, this other mechanism could also explain the reduction of QUIN-induced cell death in Bax KO mice striatum.

In summary, our results show that Bax deficiency induces a specific regulation of other Bcl-2 family members during postnatal development as well as after excitotoxicity. In the absence of Bax, Bim and Bak are regulated in a regional and temporal manner pointing to a role of these proteins in the control of cell death pathways that are active during these postnatal periods. Furthermore, Bak and Bcl-x_L are increased after QUIN-induced excitotoxicity only in Bax KO animals suggesting a possible compensatory mechanism. In contrast, Bim protein levels are similarly increased in the presence or absence of Bax indicating an important role of this protein in the initiation of the apoptotic process induced by excitotoxicity.

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REFERENCES

- Bar-Peled O., Knudson M., Korsmeyer S. J. and Rothstein J. D. (1999) Motor neuron degeneration is attenuated in bax-deficient neurons in vitro. *J. Neurosci. Res.* **55**, 542-556.
- Bouillet P., Cory S., Zhang L. C., Strasser A. and Adams J. M. (2001) Degenerative disorders caused by Bcl-2 deficiency prevented by loss of its BH3-only antagonist Bim. *Dev. Cell.* **1**, 645-653.
- Cheng E. H., Wei M. C., Weiler S., Flavell R. A., Mak T. W., Lindsten T. and Korsmeyer S. J. (2001) BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol. Cell.* **8**, 705-711.
- Chittenden T., Flemington C., Houghton A. B., Ebb R. G., Gallo G. J., Elangovan B., Chinnadurai G. and Lutz R. J. (1995) A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. *EMBO J.* **14**, 5589-5596.
- Cory S. and Adams J. M. (2002) The Bcl2 family: regulators of the cellular life-or-death switch. *Nat. Rev. Cancer* **2**, 647-656.
- Danial N. N. and Korsmeyer S. J. (2004) Cell death: critical control points. *Cell* **116**, 205-219.
- de Bilbao F., Guarin E., Nef P., Vallet P., Giannakopoulos P. and Dubois-Dauphin M. (1999) Postnatal distribution of cyp32/caspase 3 mRNA in the mouse central nervous system: an in situ hybridization study. *J. Comp. Neurol.* **409**, 339-357.
- Deckwerth T. L., Elliott J. L., Knudson C. M., Johnson E. M., Snider W. D. and Korsmeyer S. J. (1996) BAX is required for neuronal death after trophic factor deprivation and during development. *Neuron* **17**, 401-411.
- Degenhardt K., Sundararajan R., Lindsten T., Thompson C. and White E. (2002) Bax and Bak independently promote cytochrome C release from mitochondria. *J. Biol. Chem.* **277**, 14127-14134.
- Ekshyyan O. and Aw T. Y. (2004) Apoptosis in acute and chronic neurological disorders. *Front. Biosci.* **9**, 1567-1576.
- Fan H., Favero M. and Vogel M. W. (2001) Elimination of Bax expression in mice increases cerebellar purkinje cell numbers but not the number of granule cells. *J. Comp. Neurol.* **436**, 82-91.
- Ferrer I., Tortosa A., Condom E., Blanco R., Macaya A. and Planas A. (1994) Increased expression of bcl-2 immunoreactivity in the developing cerebral cortex of the rat. *Neurosci. Lett.* **179**, 13-16.
- Fishell G. and van der Kooy D. (1991) Pattern formation in the striatum: neurons with early projections to the substantia nigra survive the cell death period. *J. Comp. Neurol.* **312**, 33-42.

- Gao Y., Signore A. P., Yin W., Cao G., Yin X. M., Sun F., Luo Y., Graham S. H. and Chen J. (2005) Neuroprotection against focal ischemic brain injury by inhibition of c-Jun N-terminal kinase and attenuation of the mitochondrial apoptosis-signaling pathway. *J. Cereb. Blood Flow Metab* **25**, 694-712.
- Gibson M. E., Han B. H., Choi J., Knudson C. M., Korsmeyer S. J., Parsadanian M. and Holtzman D. M. (2001) BAX contributes to apoptotic-like death following neonatal hypoxia-ischemia: evidence for distinct apoptosis pathways. *Mol. Med.* **7**, 644-655.
- Gillardon F., Wickert H. and Zimmermann M. (1995) Up-regulation of bax and down-regulation of bcl-2 is associated with kainate-induced apoptosis in mouse brain. *Neurosci. Lett.* **192**, 85-88.
- Gilley J., Coffey P. J. and Ham J. (2003) FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. *J. Cell Biol.* **162**, 613-622.
- Gonzalez-Garcia M., Garcia I., Ding L., O'Shea S., Boise L. H., Thompson C. B. and Nunez G. (1995) bcl-x is expressed in embryonic and postnatal neural tissues and functions to prevent neuronal cell death. *Proc. Natl. Acad. Sci. U. S. A* **92**, 4304-4308.
- Green D. R. and Kroemer G. (2004) The pathophysiology of mitochondrial cell death. *Science* **305**, 626-629.
- Harris C. A. and Johnson E. M. (2001) BH3-only Bcl-2 family members are coordinately regulated by the JNK pathway and require Bax to induce apoptosis in neurons. *J. Biol. Chem.* **276**, 37754-37760.
- Hengartner M. O. (2000) The biochemistry of apoptosis. *Nature* **407**, 770-776
- Huang D. C. and Strasser A. (2000) BH3-Only proteins - Essential initiators of apoptotic cell death. *Cell* **103**, 839-842.
- Krajewska M., Mai J. K., Zapata J. M., Ashwell K. W., Schendel S. L., Reed J. C. and Krajewski S. (2002) Dynamics of expression of apoptosis-regulatory proteins Bid, Bcl-2, Bcl-X, Bax and Bak during development of murine nervous system. *Cell Death Differ.* **9**, 145-157.
- Krajewski S., Krajewska M., Shabaik A., Miyashita T., Wang H. G. and Reed J. C. (1994) Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of Bcl-2. *Am. J. Pathol.* **145**, 1323-1336.
- Krajewski S., Mai J. K., Krajewska M., Sikorska M., Mossakowski M. J. and Reed J. C. (1995) Upregulation of bax protein levels in neurons following cerebral ischemia. *J. Neurosci.* **15**, 6364-6376.
- Krajewski S., Krajewska M. and Reed J. C. (1996) Immunohistochemical analysis of in vivo patterns of Bak expression, a proapoptotic member of the Bcl-2 protein family. *Cancer Res.* **56**, 2849-2855.

- Krasnova I. N., Ladenheim B. and Cadet J. L. (2005) Amphetamine induces apoptosis of medium spiny striatal projection neurons via the mitochondria-dependent pathway. *FASEB J.* **19**, 851-853.
- Lindsten T., Ross A. J., King A., Zong W. X., Rathmell J. C., Shiels H. A., Ulrich E., Waymire K. G., Mahar P., Frauwirth K., Chen Y. F., Wei M., Eng V. M., Adelman D. M., Simon M. C., Ma A., Golden J. A., Evan G., Korsmeyer S. J., MacGregor G. R. and Thompson C. B. (2000) The combined functions of proapoptotic Bcl-2 family members Bak and Bax are essential for normal development of multiple tissues. *Mol. Cell* **6**, 1389-1399.
- Lucken-Ardjomande S. and Martinou J. C. (2005) Newcomers in the process of mitochondrial permeabilization. *J. Cell Sci.* **118**, 473-483.
- Maciejewska B., Lipowska M., Kowianski P., Domaradzka-Pytel B. and Morys J. (1998) Postnatal development of the rat striatum-a study using in situ DNA end labeling technique. *Acta Neurobiol. Exp.* **58**, 23-28.
- Martin L. J., Price A. C., McClendon K. B., Al-Abdulla N. A., Subramaniam J. R., Wong P. C. and Liu Z. (2003) Early events of target deprivation/axotomy-induced neuronal apoptosis in vivo: oxidative stress, DNA damage, p53 phosphorylation and subcellular redistribution of death proteins. *J. Neurochem.* **85**, 234-247.
- Merry D. E., Veis D. J., Hickey W. F. and Korsmeyer S. J. (1994) bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. *Development* **120**, 301-311.
- Merry D. E. and Korsmeyer S. J. (1997) Bcl-2 gene family in the nervous system. *Annu. Rev. Neurosci.* **20**, 245-267.
- Mikhailov V., Mikhailova M., Degenhardt K., Venkatachalam M. A., White E. and Saikumar P. (2003) Association of Bax and Bak homo-oligomers in mitochondria. Bax requirement for Bak reorganization and cytochrome c release. *J. Biol. Chem.* **278**, 5367-5376.
- Napankangas U., Lindqvist N., Lindholm D. and Hallbook F. (2003) Rat retinal ganglion cells upregulate the pro-apoptotic BH3-only protein Bim after optic nerve transection. *Mol. Brain Res.* **120**, 30-37.
- Mooney S. M. and Miller M. W. (2000) Expression of bcl-2, bax, and caspase-3 in the brain of the developing rat. *Brain Res. Dev. Brain Res.* **123**, 103-117.
- Oltvai Z. N., Millman C. L. and Korsmeyer S. J. (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74**, 609-619.
- Oorschot D. E. (1996) Total number of neurons in the neostriatal, pallidal, subthalamic, and substantia nigral nuclei of the rat basal ganglia: a stereological study using the cavalieri and optical disector methods. *J. Comp. Neurol.* **366**, 580-599.

- O'Reilly L. A., Cullen L., Visvader J., Lindeman G. J., Print C., Bath M. L., Huang D. C. and Strasser A. (2000) The proapoptotic BH3-only protein bim is expressed in hematopoietic, epithelial, neuronal, and germ cells. *Am. J. Pathol.* **157**, 449-461.
- Okuno S., Saito A., Hayashi T. and Chan P. H. (2004) The c-Jun N-terminal protein kinase signaling pathway mediates Bax activation and subsequent neuronal apoptosis through interaction with Bim after transient focal cerebral ischemia. *J. Neurosci.* **24**, 7879-7887.
- Parsadanian A. S., Cheng Y., Keller-Peck C. R., Holtzman D. M. and Snider W. D. (1998) Bcl-xL is an antiapoptotic regulator for postnatal CNS neurons. *J. Neurosci.* **18**, 1009-1019.
- Perez-Navarro E., Canudas A. M., Akerud P., Alberch J. and Arenas E. (2000) Brain-Derived Neurotrophic Factor, Neurotrophin-3, and Neurotrophin-4/5 prevent the death of striatal projection neurons in a rodent model of Huntington's disease. *J. Neurochem.* **75**, 2190-2199.
- Perez-Navarro E., Gavaldà N., Gratacos E. and Alberch J. (2005) Brain-derived neurotrophic factor prevents changes in Bcl-2 family members and caspase-3 activation induced by excitotoxicity in the striatum. *J. Neurochem.* **92**, 678-691.
- Putcha G. V., Deshmukh M. and Johnson E. M., Jr. (2000) Inhibition of apoptotic signaling cascades causes loss of trophic factor dependence during neuronal maturation. *J. Cell Biol.* **149**, 1011-1018.
- Putcha G. V., Moulder K. L., Golden J. P., Bouillet P., Adams J. A., Strasser A. and Johnson E. M. (2001) Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis. *Neuron* **29**, 615-628.
- Puthalakath H., Huang D. C., O'Reilly L. A., King S. M. and Strasser A. (1999) The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol. Cell.* **3**, 287-296.
- Puthalakath H. and Strasser A. (2002) Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. *Cell. Death. Differ.* **9**, 505-512.
- Sattler M., Liang H., Nettesheim D., Meadows R. P., Harlan J. E., Eberstadt M., Yoon H. S., Shuker S. B., Chang B. S., Minn A. J., Thompson C. B. and Fesik S. W. (1997) Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. *Science* **275**, 983-986.
- Shimohama S., Fujimoto S., Sumida Y. and Tanino H. (1998) Differential expression of rat brain bcl-2 family proteins in development and aging. *Biochem. Biophys. Res. Commun.* **252**, 92-96.
- Shindler K. S., Latham C. B. and Roth K. A. (1998) Bax deficiency prevents the increased cell death of immature neurons in bcl-x-deficient mice. *J. Neurosci.* **17**, 3112-3119.
- Shinoda S., Schindler C. K., Meller R., So N. K., Araki T., Yamamoto A., Lan J. Q., Taki W., Simon R. P. and Henshall D. C. (2004) Bim regulation may determine

- hippocampal vulnerability after injurious seizures and in temporal lobe epilepsy. *J. Clin. Invest.* **113**, 1059-1068.
- Spreafico R., Frassoni C., Arcelli P., Selvaggio M. and De B. S. (1995) In situ labeling of apoptotic cell death in the cerebral cortex and thalamus of rats during development. *J. Comp. Neurol.* **363**, 281-295.
- Vekrellis K., McCarthy M. J., Watson A., Whitfield J., Rubin L. L. and Ham J. (1997) Bax promotes neuronal cell death and is downregulated during the development of the nervous system. *Development* **124**, 1239-1249.
- Vila M., Jackson-Lewis V., Vukosavic S., Djaldetti R., Liberatore G., Offen D., Korsmeyer S. J. and Przedborski S. (2001) Bax ablation prevents dopaminergic neurodegeneration in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 2837-2842.
- Vila M. and Przedborski S. (2003) Targeting programmed cell death in neurodegenerative diseases. *Nat. Rev. Neurosci.* **4**, 365-375.
- Vis J. C., Verbeek M. M., De Waal R. M., Ten Donkelaar H. J. and Kremer B. (2001) The mitochondrial toxin 3-nitropropionic acid induces differential expression patterns of apoptosis-related markers in rat striatum. *Neuropathol. Appl. Neurobiol.* **27**, 68-76.
- Wei M. C., Zong W. X., Cheng E. H. Y., Lindsten T., Panoutsakopoulou V., Ross A. J., Roth K. A., MacGregor G. R., Thompson C. B. and Korsmeyer S. J. (2001) Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death. *Science* **292**, 727-730.
- White F. A., Keller-Peck C. R., Knudson C. M., Korsmeyer S. J. and Snider W. D. (1998) Widespread elimination of naturally occurring neuronal death in Bax-deficient mice. *J. Neurosci.* **18**, 1428-1439.
- Whitfield J., Neame S. J., Paquet L., Bernard O. and Ham J. (2001) Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. *Neuron* **29**, 629-643.
- Willis S. N., Chen L., Dewson G., Wei A., Naik E., Fletcher J. I., Adams J. M. and Huang D. C. (2005) Proapoptotic bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev.* **19**, 1294-1305.
- Xiang H., Kinoshita Y., Knudson C. M., Korsmeyer S. J., Schwartzkroin P. A. and Morrison R. S. (1998) Bax involvement in p53-mediated neuronal cell death. *J. Neurosci.* **18**, 1363-1373.
- Zong W. X., Lindsten T., Ross A. J., MacGregor G. R. and Thompson C. B. (2001) BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. *Genes Dev.* **15**, 1481-1486.