DEPARTAMENT DE
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MECANISMOS INTRACELULARES DE SUPERVIVENCIA Y MUERTE NEURONAL EN MODELOS EXCITOTÓXICOS Y TRANSGÉNICOS DE LA ENFERMEDAD DE HUNTINGTON

Tesis presentada por Juan Manuel García Martínez

para optar al título de Doctor por la Universidad de Barcelona

III.- RESULTADOS

PRIMER TRABAJO: *"Glial Cell Line-Derived Neurotrophic Factor Promotes the Arborization of Cultured Striatal Neurons Through the p42/p44 Mitogen-Activated Protein Kinase Pathway GDNF".*

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OBJETIVO 1.- Caracterización de los efectos neurotróficos del GDNF sobre las neuronas estriatales en cultivo.

1.1.- Análisis de las vías de señalización intracelular activadas por el GDNF.

1.2.- Estudio del efecto del GDNF sobre la maduración y diferenciación de las neuronas GABAérgicas estriatales.

En el núcleo estriado, las neuronas de proyección representan el 90% de la población total. Estas neuronas son GABAérgicas y expresan el marcador calbindina en sus últimas etapas de maduración. Se ha descrito como los diferentes factores neurotróficos pueden promover la supervivencia y la maduración de las neuronas de proyección, aunque se conoce poco acerca de los efectos específicos mediados por el GDNF. Así, para cumplir con el primer objetivo, analizamos las vías intracelulares implicadas en la señalización del GDNF en las neuronas estriatales en cultivo. Nuestro propósito era determinar los efectos que ejerce este factor neurotrófico sobre la supervivencia y diferenciación de esta población neuronal. Las neuronas GABAérgicas de proyección degeneran de manera específica en la enfermedad de Huntington, y con este trabajo se complementan estudios previos de nuestro laboratorio con la intención de evaluar el potencial neuroprotector de los diferentes factores neurotróficos.

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Glial Cell Line-Derived Neurotrophic Factor Promotes the Arborization of Cultured Striatal Neurons Through the p42/p44 Mitogen-Activated Protein Kinase Pathway

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Glial cell line-derived neurotrophic factor (GDNF) promotes the survival or differentiation of several types of neurons. This study examines GDNF-induced signal transduction and biological effects in cultured striatal neurons. Results show that GDNF addition to striatal cultures transiently increased the protein levels of phosphorylated p42/p44, but did not change the levels of phosphorylated Akt. GDNF effects on phosphorylated p42/p44 levels were blocked by the mitogen-activated protein kinase (MAPK) pathway specific inhibitors (PD98059 and U0126). Activation of the p42/p44 MAPK pathway by GDNF led to an increase in the degree of dendritic arborization and axon length of both GABA- and calbindin-positive neurons but had no effect on their survival and maturation. These GDNF-mediated effects were suppressed in the presence of the inhibitor of the MAPK pathway (PD98059). Furthermore, the addition of the phosphatidylinositol 3-kinase pathway specific inhibitor (LY294002) blocked GDNF-mediated striatal cell differentiation suggesting that the basal activity of this pathway is needed for the effects of GDNF. Our results indicate that treatment of cultured striatal cells with GDNF specifically activates the p42/p44 MAPK pathway, leading to an increase in the arborization of GABA- and calbindin-positive neurons.

Key words: calbindin; GABA; differentiation; PI3-K

Neurotrophic factors are essential proteins for the regulation of neuronal survival, growth and differentiation during development (Baloh et al., 2000; Huang and Reichardt, 2001; Davies, 2003). Most of them stimulate a receptor tyrosine kinase, which activates several well-defined signaling cascades (Airaksinen and Saarma, 2002; Huang and Reichardt, 2003; Segal, 2003). The receptor tyrosine kinase Ret (Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996) is an important component in the signaling cascade activated by members of the glial cell line-derived neurotrophic factor (GDNF) family, a group of structurally and functionally related polypeptides. This receptor is activated only if GDNF ligands are bound to an accessory protein linked to the plasma membrane by a glycosyl phosphatidylinositol anchor named GDNF family receptor α (GFRα; Airaksinen and Saarma, 2002). Stimulation of Ret initiates several downstream intracellular pathways, of which the phosphatidylinositol 3-kinase (PI3-K) and the p42/p44 mitogen-activated protein kinase (MAPK) pathways are the most extensively studied (Airaksinen and Saarma, 2002). The activation of these pathways may promote neuronal survival or differentiation (Pong et al., 1998; Van Weering and Bos, 1998; Soler et al., 1999; Coulpier et al., 2002; Pelicci et al., 2002).

GDNF, the first member of the GDNF family to be discovered, was initially characterized as a neurotrophic factor for midbrain dopaminergic neurons (Lin et al., 1993). In agreement with its role on nigrostriatal dopaminergic neurons, GDNF is highly expressed in the striatum during development (Schaar et al., 1993; Choi-Lundberg and Bohn, 1995; Golden et al., 1999). The GDNF receptors, Ret and GFRα1, are also expressed

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by striatal neurons (Golden et al., 1999; Pérez-Navarro et al., 1999; Marco et al., 2002; Cho et al., 2004) suggesting that these neurons can also respond to GDNF (Alberch et al., 2004). Indeed, GDNF protects striatal neurons against excitotoxicity (Pérez-Navarro et al., 1996, 1999; Araujo and Hilt, 1997; Gratacos et al., 2001a; Kells et al., 2004) or 3-nitropropionic acid lesion (Araujo and Hilt, 1998).

In the striatum, projection neurons account for 90% of the overall population (Smith et al., 1990). They are GABAergic and they also express calbindin in a late stage of maturation (Liu and Graybiel, 1992). Several neurotrophic factors have been shown to promote the survival or maturation of striatal GABAergic neurons in vitro (Mizuno et al., 1994; Ventimiglia et al., 1995; Ivkovic et al., 1997; Gratacos et al., 2001b,c; Gavalda et al., 2004) but very little is known about the biological effects of GDNF (Humpel et al., 1996; Farkas et al., 1997). Furthermore, there are no data about the intracellular signaling pathways activated by this neurotrophic factor in striatal neurons. We examined whether GDNF activates the p42/p44 MAPK or PI3-K pathways, and the functional meaning of this activation in the survival, maturation or differentiation of striatal GABAergic neurons in vitro.

MATERIALS AND METHODS

Cell Culture

Animal handling procedures were approved by the Local Committee (99/1 University of Barcelona) and the Generalitat de Catalunya (1094/99), in accordance with the Directive 86/609/EU of the European Commission. Certified time-pregnant Sprague-Dawley dams (Charles River Laboratories, France) were deeply anesthetized on gestational day (GD) 19, fetuses were rapidly removed from the uterus and striatal cells were obtained as described elsewhere (Gratacos et al., 2001b). Cells were plated at a density of 50,000 cells/cm^2 onto 24-well plates or 60-mm culture dishes, which were precoated with 0.1 mg/ml poly-D lysine (Sigma Chemical Co., St. Louis, MO), for morphological or Western blot analysis, respectively. Eagle's minimum essential medium (MEM; Gibco-BRL, Renfrewshire, Scotland, UK) supplemented with B-27 (Gibco-BRL) was used to grow the cells in serum-free conditions. To study the activation of PI3-K and p42/p44 MAPK pathways, medium was removed at 3 or 7 days in vitro (DIV) and replaced by N2-supplemented medium to deprive cells for 3 hr. GDNF (Peprotech EC Ltd., London, UK) was added to the cultures and Akt and p42/p44 phosphorylation was examined at different time points. Protein levels of phosphorylated CREB were examined 5 min after GDNF addition to the cultures following the same protocol. In another set of experiments, cultures were treated with various inhibitors, such as PD98059 (25 or 50 μM; Calbiochem, San Diego, CA) (Dudley et al., 1995), U0126 (5 or 10 μM; Calbiochem) (Favata et al., 1998) or LY294002 (25 or 50 μM; Biomol Research Laboratories, Plymouth Meeting, PA) (Vlahos et al., 1994). They were dissolved in N2-supplemented medium containing bovine serum albumin (6.6 mg/ml; Sigma), and added to cell cultures 1 hr before GDNF treatment. To examine the involvement of lipid rafts in GDNF signaling, cultures were treated with 2-hydroxypropyl-β cyclodextrin (5 mM; Sigma) during 10 min before GDNF addition. For morphological analysis, MEM supplemented with B-27 was used to grow the cells and at 3DIV GDNF (50 ng/ml) was added alone or in combination with LY294002 or PD98059. Two days after treatments the medium was removed and replaced by MEM supplemented with B-27 until 7DIV, when the cultures were fixed. Plated cell cultures were maintained in an incubator with 5% CO_2 at 37°C.

Western Blot Analysis

After GDNF exposure, cells were rinsed rapidly in ice-cold phosphate-buffered saline (PBS), and lysed with buffer as described elsewhere (Gavalda et al., 2004). Membranes were incubated overnight at 4°C with antibodies against phospho-p42/p44 (1:5,000; Cell Signaling Technology, Beverly, MA), phospho-Akt (1:2,000; Cell Signaling Technology) or phospho-CREB (1:1,000; Cell Signaling Technology). To standardize total protein content in each lane, membranes were incubated for 1 hr at room temperature (r.t.) with antibodies against panERK (1:5,000; BD Transduction Laboratories), pan-Akt (1:500; Cell Signaling Technology), or CREB (1:1,000; Cell Signaling Technology). After addition of the corresponding secondary antibody conjugated to horseradish peroxidase (1:2,000; Promega, Madison, WI), membranes were developed using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA). Western blot replicates were scanned and quantified using the Phoretix 1D Gel Analysis (Phoretix International Ltd., Newcastle, UK).

Immunocytochemistry

Striatal cultures were fixed with 4% paraformaldehyde for 1 hr at r.t., followed by three rinses in PBS. Cells were then preincubated for 15–30 min with PBS containing 0.3% Triton X-100 (Sigma) and 30% normal horse serum (Gibco-BRL) at r.t. Cultures were then incubated overnight at 4°C with antibodies directed against calbindinD28K (1:10,000; Swant) GABA (1:1000; Sigma), GFR-α1 (1:100; Serotec, Oxford, UK), or Ret (C-20, 1:250; Santa Cruz Biotechnology) diluted in PBS containing 0.3% Triton X-100 and 5% normal horse serum. Cells were then incubated in biotinylated secondary antibodies, then with avidin-biotin complex (Pierce ABC Kit) and finally developed with 0.05% diaminobenzidine and 0.02% H_2O_2.

Detection of Cell Death

At 3DIV, cultures were treated with GDNF (50 ng/ml) and dying neurons were detected 2 days later. Cells were fixed with 4% paraformaldehyde for 1 hr at r.t., followed by three rinses in PBS. Neurons were incubated with DAPI (1:100; Sigma) for 5 min and then rinsed twice with PBS.

Quantitative Analysis of Cell Cultures

Total cell number, GABAergic neurons, calbindin-positive neurons and pyknotic/fragmented nuclei stained by DAPI

were counted in 20 fields at 200×. Cell number was analyzed in four to six wells per condition and in four independent experiments. Morphological parameters were assessed using a PC-Image analysis system from Foster Findlay on a computer attached to an Olympus microscope. GABA- and calbindin-positive neurons (60/condition) were chosen at random and traced in a phase-contrast image using the mouse hook up. Total and soma area, perimeter and degree of arborization ($Perimeter^2/4\pi Area$) were determined as described by Fujita et al. (1996). Axon length was also measured, considering the axon as the longest emerging neurite from the soma, as previously described (Gratacos et al., 2001b). Statistical significance was assessed by ANOVA followed by the L.S.D. post-hoc test.

RESULTS

GDNF Specifically Activates the p42/p44 MAPK Pathway

To identify which intracellular pathways were activated by GDNF in striatal neurons, medium was removed on 3DIV and replaced by N2-supplemented medium 3 hr before GDNF (50 ng/ml) addition. Cells were deprived during this time period because in basal conditions PI3-K and p42/p44 MAPK pathways were highly activated (data not shown). Five minutes after GDNF treatment phospho-p42/p44 levels rose sharply (by 2-fold, Fig. 1A). In contrast, levels of phospho-Akt were not affected by GDNF at any time (Fig. 1C). After cell culture deprivation, however, basal phospho-Akt levels increased in both control and GDNF-treated cultures (Fig. 1C), whereas phospho-p42/p44 levels did not change (Fig. 1B). The same membranes were reproved for total Akt and ERK, respectively, showing that total levels of the protein were not modified (Fig. 1).

We next examined whether activation of the PI3-K pathway by GDNF was dose dependent. Addition of 100 ng/ml of GDNF did not affect phospho-Akt levels (GDNF 50 ng/ml: 106 ± 20; GDNF 100 ng/ml: 82 ± 10; results obtained 5 min after GDNF addition and expressed as a percentage of phospho-Akt control values). In contrast, p42/p44 phosphorylation levels were higher than after treatment with 50 ng/ml of GDNF (GDNF 50 ng/ml: phospho-p44, 213 ± 58; phospho-p42, 625 ± 89; GDNF 100 ng/ml: phospho-p44, 389 ± 28; phospho-p42, 1035 ± 117; results obtained 5 min after GDNF addition and expressed as a percentage of phospho-p44 levels in control condition).

Because we did not observe PI3-K pathway activation after GDNF addition at any of the doses used, we asked whether this effect was related to the levels of GFRα1 and Ret receptors expressed by striatal neurons. Both GFRα1 (Fig. 2A) and Ret (Fig. 2B) receptors were detected by immunocytochemistry at 3DIV, although Ret was expressed at very low levels. At 7DIV, the expression levels of Ret, but not GFRα1, were increased (Fig. 2C,D). We analyzed p42/p44 MAPK and PI3-K pathways activation after GDNF (50 ng/ml) addition at 7DIV. As shown in Figure 2E, the response of striatal cells after GDNF treatment at 7DIV was similar to that observed at 3DIV (Fig. 1A).

The involvement of the lipid rafts in the intracellular signaling activated by GDNF in striatal cells was also examined. Striatal cells, on 3DIV, were treated with 2-hydroxypropyl-β cyclodextrin, a cholesterol-binding agent that disperses lipid rafts (Pol et al., 2005), 10 min before stimulation with neurotrophic factors. As shown in Figure 3, cholesterol-depleted cells displayed attenuated phosphorylation of p42/p44 in response to GDNF, but not to BDNF (10 ng/ml), stimulation compared to non treated cells.

To further characterize the activation of the p42/p44 MAPK pathway by GDNF in striatal cells, cultures were treated for 1 hr with specific inhibitors before the addition of the trophic factor (50 ng/ml). Pretreatment with PD98059 (25–50 μM) or U0126 (5–10 μM) reduced GDNF-induced p42/p44 phosphorylation (Fig. 4A). Furthermore, PD98059 (50 μM) blocked the increase in phospho-CREB (a downstream target of p42/p44 MAPK) protein levels induced by GDNF (Fig. 4B). We also analyzed the effect of PI3-K inhibitors in p42/p44 activation by GDNF. In cultures treated with LY294002 (25 μM) alone basal levels of phospho-Akt decreased but phospho-p42/p44 was unaffected (Fig. 4C), showing that this inhibitor selectively blocks PI3-K activation. Abrogation of PI3-K by pretreatment of cells with LY294002 (25 μM) did not modify GDNF-induced phosphorylation of p42/p44 (Fig. 4B), but addition of a higher dose of LY294002 (50 μM) slightly inhibited (by 30%) phospho-p42/p44 levels (Fig. 4B).

GDNF Treatment Has No Effect on Neuronal Survival

The next step was to investigate the biological effects resulting from GDNF-induced activation of the p42/p44 MAPK pathway in striatal neurons. The percentage of dying cells was similar in control (29 ± 1%) and in GDNF-treated (23 ± 4%) cultures. Similarly, the total number of cells at 7DIV was not modified by GDNF (50 ng/ml) addition at 3DIV (Control: 18,865 ± 2,609 cells/cm^2; GDNF: 17,287 ± 2,273 cells/cm^2).

GDNF-Mediated Stimulation of the p42/p44 MAPK Pathway Promotes the Arborization, But Not the Maturation, of GABA- and Calbindin-Positive Striatal Neurons

We studied whether GDNF induces GABA and calbindin phenotypes, and the differentiation of these neuronal populations. The number of GABA-positive neurons was not modified by GDNF (Control: 13,659 ± 3,558 cells/cm^2; GDNF-treated: 13,248 ± 3,291 cells/cm^2). Similarly, no differences were detected between the number of calbindin-positive neurons in control (684 ± 108 cells/cm^2) and in GDNF-treated cultures (694 ± 119 cells/cm^2).

Morphological analysis was carried out to investigate the involvement of GDNF in the differentiation of

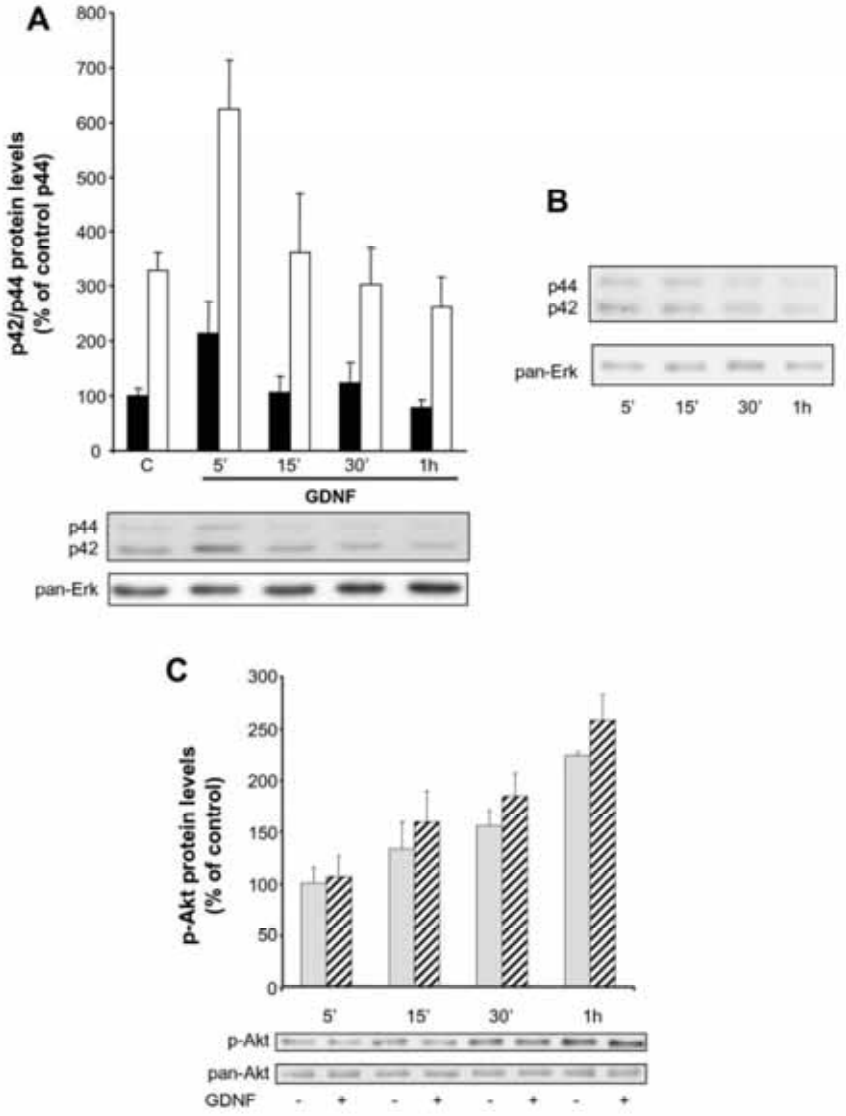
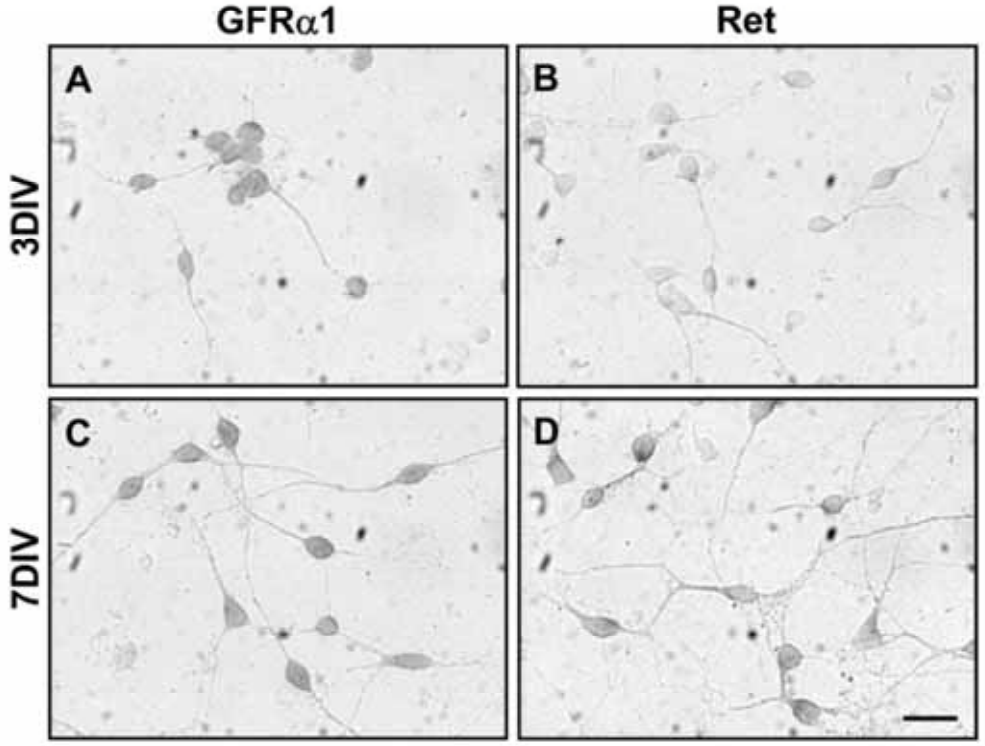


Fig. 1. GDNF treatment activates the p42/p44 MAPK but not the PI3-K pathway. GDNF (50 ng/ml) was added to cultures at 3DIV, and p42/p44 and Akt phosphorylation were examined by Western blot at different time points. **A:** Bars showing phospho-p42/p44 protein levels. Results were obtained from densitometric analysis ($n = 4$) and expressed as a percentage of phospho-p44 protein levels in control condition. p42, open bars; p44, filled bars. Immunoblots (obtained from a representative experiment) show the increase in phospho-p42/p44 protein levels five minutes after GDNF addition. **B:** In the absence of GDNF stimulation, phospho-p42/p44 protein levels did not change at different time points after deprivation. **C:** Bars showing phospho-Akt protein levels. Results obtained from densitometric analysis ($n = 4$) were expressed as a percentage of control (gray bars); GDNF-treated, hatched bars. Immunoblot show a representative experiment.

GABA- and calbindin-positive neurons. GDNF treatment increased the total area, perimeter, axon length and degree of arborization of both GABA- (Fig. 5) and calbindin-positive (Fig. 6) neurons, without modifying the soma area. The effects on the degree of arborization were higher in the calbindin-positive population (compare Figs. 5 and 6). These morphological parameters were also analyzed in the presence of the inhibitor PD98059 (50 μM). The addition of PD98059 alone did not affect the differentiation of GABA- (axon length, in μm: 40 ± 1; degree of arborization: 14 ± 1) or calbindin-positive neurons (axon length, in μm: 53 ± 1; degree of arborization: 20 ± 1). In contrast, addition of PD98059 in combination with GDNF blocked the effects of the neurotrophic factor on the differentiation of both GABA- and calbindin-positive neurons (Figs. 5,6, respectively). In this condition, all the parameters analyzed were the same as control. The differentiation of GABA- and calbindin-positive neurons after treatment with LY294002 alone was similar to that observed in control (axon length in μm: 45 ± 2 and 58 ± 4; degree of arborization: 16 ± 2 and 22 ± 1, for GABA- and calbindin-positive neurons, respectively). Unexpectedly, GDNF-mediated effects on the differentiation of GABA- and calbindin-positive neurons were inhibited in the presence of LY294002 (Figs. 5,6).



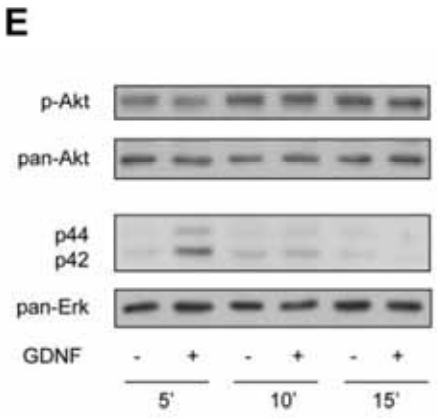


Fig. 2. Ret expression increases with time in culture but GDNF fails to activate the PI3-K pathway. GFRα1 (**A,C**) and Ret (**B,D**) expression was examined by immunocytochemistry at 3 (A,B) and 7 (C,D) DIV. Scale bar = 50 μm. Ret, but not GFRα1, expression was increased at 7DIV. **E:** Immunoblot obtained from a representative experiment showing phospho-Akt and phospho-p42/p44 levels at different time points after GDNF addition (50 ng/ml) to striatal cultured cells at 7DIV. Phospho-p42/p44, but not phospho-Akt, protein levels increased 5 min after GDNF treatment, similarly to that observed at 3DIV.

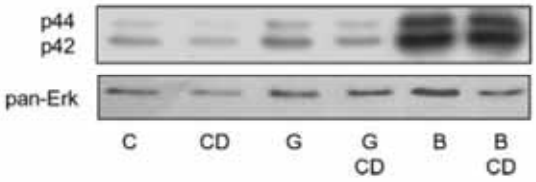


Fig. 3. Disruption of lipid raft integrity attenuates GDNF-induced signaling. Cultured striatal cells (3DIV) were treated with 2-hydroxypropyl-β cyclodextrin to remove cholesterol from the plasma membrane and disperse lipid rafts. GDNF-, but not BDNF-induced increase in phospho-p42/p44 protein levels was attenuated in cultured striatal cells treated with 2-hydroxypropyl-β cyclodextrin. Immunoblot show a representative experiment. Similar results were obtained in three independent experiments. C, control non-treated cells; CD, cells treated with 2-hydroxypropyl-β cyclodextrin (2 mM) during 15 min; G, cells treated with GDNF (50 ng/ml) during 5 min; G/CD, cells treated with 2-hydroxypropyl-β cyclodextrin plus GDNF; B, cells treated with BDNF (10 ng/ml) during 5 min; B/CD, cells treated with 2-hydroxypropyl-β cyclodextrin plus BDNF.

DISCUSSION

This study shows that GDNF specifically activates the p42/p44 MAPK pathway in cultured striatal cells. This activation leads to biological effects as GDNF treatment increases the degree of arborization and axon length in both GABA- and calbindin-positive striatal neurons. Although GDNF only activates the p42/p44 MAPK pathway, its biological effects are blocked in the presence of p42/p44 MAPK (PD98059) or PI3-K (LY294002) pathway specific inhibitors.

GDNF promotes neuronal survival (Henderson et al., 1994; Oppenheim et al., 1995; Ha et al., 1996; Price et al., 1996; Burke et al., 1998) and morphological differentiation (Mount et al., 1995; Price et al., 1996; Widmer et al., 2000; Holm et al., 2002) depending on the neuronal type examined. In our culture conditions, GDNF increased the degree of arborization of both GABA- and calbindin-positive neurons without affecting either neuronal survival or maturation. We also show that GDNF only activated the p42/p44 MAPK pathway. Consistent with our results, the activation of the p42/p44 MAPK pathway induced by GDNF has been implicated mainly in neuronal differentiation (Chen et al., 2001; Park et al., 2005) whereas the activation of the PI3K pathway has been related to survival (Miller et al., 1997; Soler et al., 1999; Encinas et al., 2001) and differentiation (van Weering and Bos, 1997; Pong et al., 1998). We suggest that GDNF-induced neuronal differentiation of striatal neurons is mediated by the activation of the p42/p44 MAPK pathway.

In our culture conditions, we did not observe PI3-K pathway activation after GDNF addition. It has been shown previously that GFRα1, in the absence of Ret, triggers the phosphorylation of p42/p44 MAPK and phospholipase Cγ (Poteryaev et al., 1999). We examined the presence of both receptors in cultured striatal neurons. Our results showed that both Ret and GFRα1 were expressed in striatal cultured neurons with higher levels of Ret at 7DIV than at 3DIV. When GDNF signaling was examined at 7DIV, however, we obtained similar response to that observed at 3DIV suggesting that other mechanisms, different to the relative abundance of the receptors, could account for the absence of PI3-K pathway activation. One important aspect for GDNF signal transduction is the recruitment of Ret to lipid rafts (Paratcha and Ibanez, 2002). These are regions within plasma membranes rich in sphingolipids and cholesterol that are important for cellular signaling (Simons and Toomre, 2000). We have observed that disruption of lipid rafts by using 2-hydroxypropyl-β-cyclodextrin, which depletes cholesterol from plasma membranes (Pol et al., 2005), blocked the p42/p44 phosphorylation induced by GDNF, suggesting an important role for lipid rafts in Ret-induced signaling on striatal neurons. However, activated Ret can also be detected outside the lipid rafts (Paratcha and Ibañez, 2002). Interestingly, activated Ret inside the lipid rafts triggers the signal through binding to FRS2 whereas outside the rafts the signal is triggered through binding to SHC (Paratcha et al., 2001). Furthermore, it has been shown previously that FRS2 is involved mainly in the activation of the p42/p44 MAPK but not the PI3-K pathway (Kurokawa et al., 2001). Therefore, it is tempting to speculate that in our system, Ret signals inside the rafts through binding to FRS2 triggering the activation only of the p42/p44 MAPK pathway, although more experiments are needed to confirm this hypothesis.

Our results show that GDNF did not affect the number of calbindin-positive neurons. Previous studies have reported that treatment of striatal neurons with another trophic factor, BDNF, increases the number of calbindin-positive neurons (Gavalda et al., 2004). This BDNF-mediated effect depends on the activation of PI3-K and p42/p44 MAPK pathways (Gavalda et al., 2004). We can suggest that GDNF did not induce the calbindin phenotype, as it did not activate the PI3-K pathway in striatal neurons. Furthermore, both GDNF (present results) and BDNF (Gavalda et al., 2004) treatment increased the degree of arborization of GABA- and calbindin-positive neurons, but the effects of BDNF were higher. Because BDNF-induced neuronal differentiation was abolished in the presence of p42/p44 MAPK or PI3-K inhibitors, taken together our results could implicate PI3-K in neuronal differentiation. However, levels of phospho-p42/p44 after BDNF addition are higher and more sustained (even 7 days after treatment) (Gavalda et al., 2004) than after GDNF treatment (only at 5 min, present results). The strength and duration of the MAPK pathway activation may also be critical for these biological effects as has been previously described in other models (Mariathasan et al., 2001; Chang et al., 2003; Rossler et al., 2004; Whitehurst et al., 2004).

GDNF-induced p42/p44 MAPK pathway activation was blocked in the presence of the specific inhibitors PD98059 and U0126. As expected, treatment with PD98059 also prevented GDNF-mediated biological

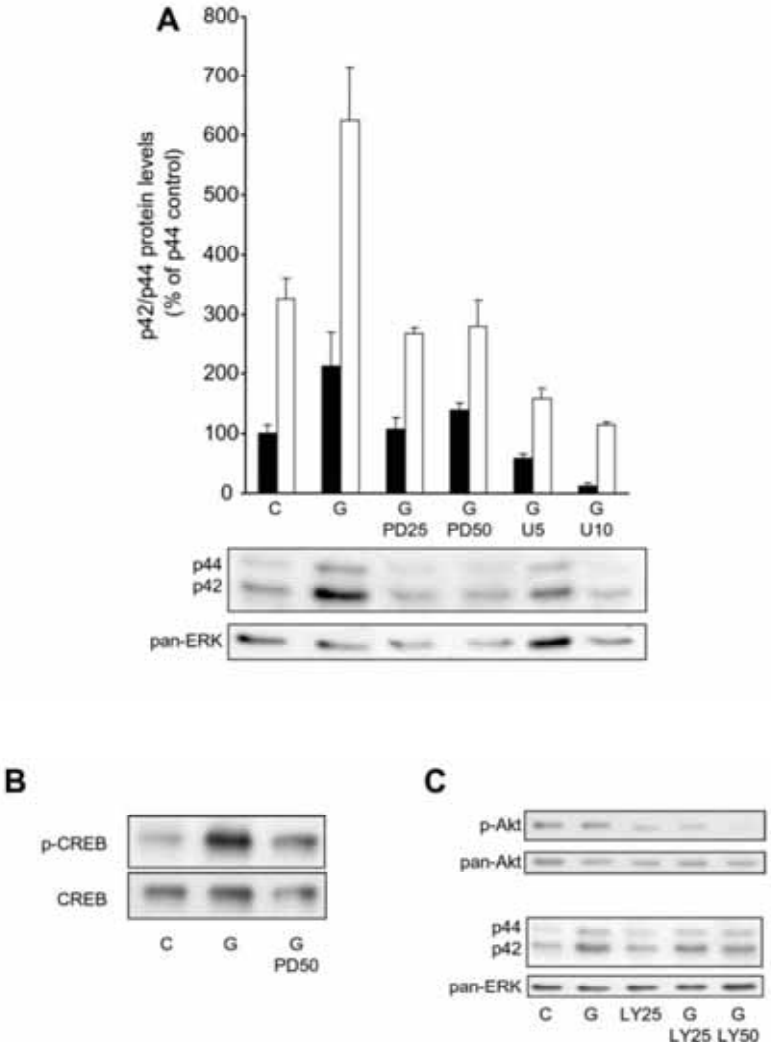
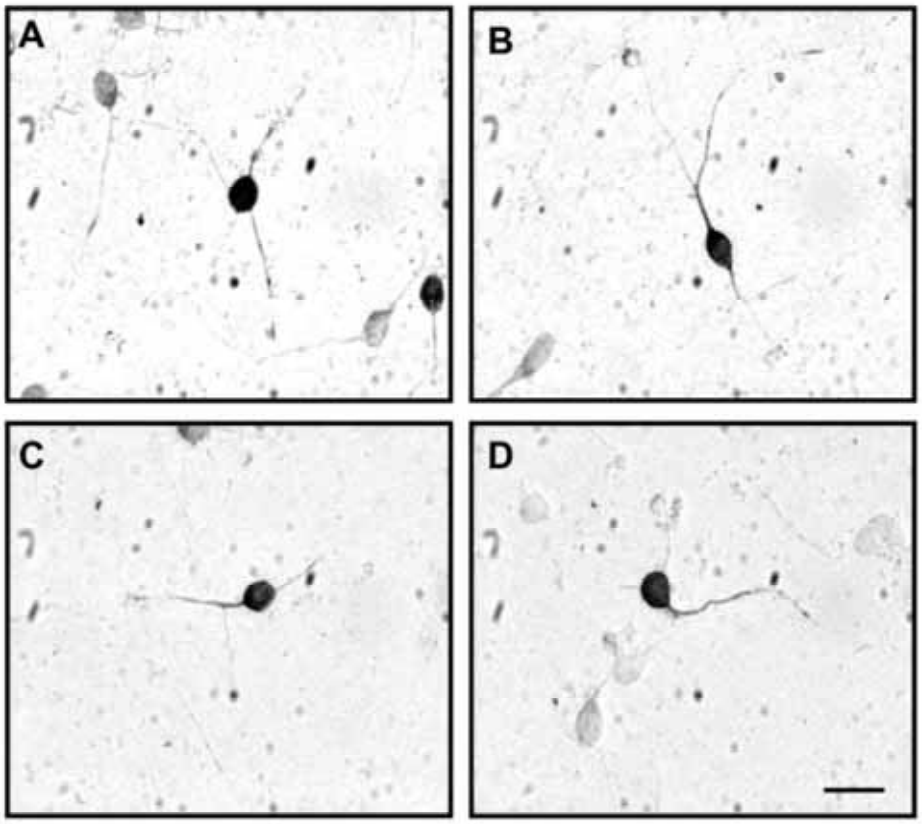


Fig. 4. GDNF-induced activation of the p42/p44 MAPK pathway is blocked by treatment with inhibitors of p42/p44 and PI3-K pathways. Cultures were treated with inhibitors for 1 hr before the addition of GDNF (50 ng/ml). Phospho-p42/p44, phospho-Akt and phospho-CREB levels were measured by Western blot at 5 min after GDNF addition. **A:** Figure showing the blockade of GDNF-induced increase in phospho-p42/p44 by treatment with specific inhibitors of the p42/p44 MAPK pathway. Results obtained from densitometric analysis ($n = 4$) were expressed as a percentage of phospho-p44 protein levels in control condition. p42, open bars; p44, filled bars; PD25, PD98059 25 μM; PD50, PD98059 50μM; U5, U0126 5 μM; U10, U0126 10 μM. Immunoblot was obtained from a representative experiment. **B:** Immunoblot showing increased levels of phospho-CREB (p-CREB) protein, 5 min after GDNF addition (G). This effect was attenuated by treatment with the inhibitor of the p42/p44 MAPK pathway (PD98059; 50μM; G/PD50). **C:** Immunoblots showing the inhibition of phospho-Akt basal levels by treatment with the PI3-K pathway specific inhibitor LY294002 25 μM (LY25), and the blockade of increased phospho-p42/p44 levels induced by GDNF by treatment with 25 μM LY294002 (G/LY25) and 50 μM LY294002 (G/LY50). C, control; G, GDNF (50 ng/ml).

effects. However, our findings also showed that treatment with LY294002, a specific inhibitor of the PI3-K pathway, blocked the biological effects mediated by GDNF. This result could not be attributed to the type of cross-talk between p42/p44 MAPK and PI3-K pathways previously described in striatal cultures (Stropollo et al., 2001; Fuller et al., 2001; Perkinton et al., 2002) because GDNF failed to produce a direct activation of the PI3-K pathway, and the dose of LY294002 (25 μM) used to analyze the biological effects did not inhibit GDNF-induced p42/p44 MAPK pathway activation. Furthermore, treatment with LY294002 blocked basal levels of phospho-Akt, underscoring that it is the basal activity of the PI3K pathway that is required for GDNF to exert its trophic effects on striatal neurons. Consistent with our data, it has been previously shown that weak stimulation, but not strong stimulation, of p42/p44 MAPK pathway could be dependent on the basal PI3-K pathway activity (Duckworth and Cantley, 1997; Wennström and Downward, 1999). In the present culture conditions, phospho-Akt, but not phospho-p42/p44 levels, gradually increased after changing the culture medium indicating that this pathway is important for neuronal survival, as previously described (Dudek et al., 1997; Miller et al., 1997; Soler et al., 1999; Kuruvilla et al., 2000; Gavalda et al., 2004).

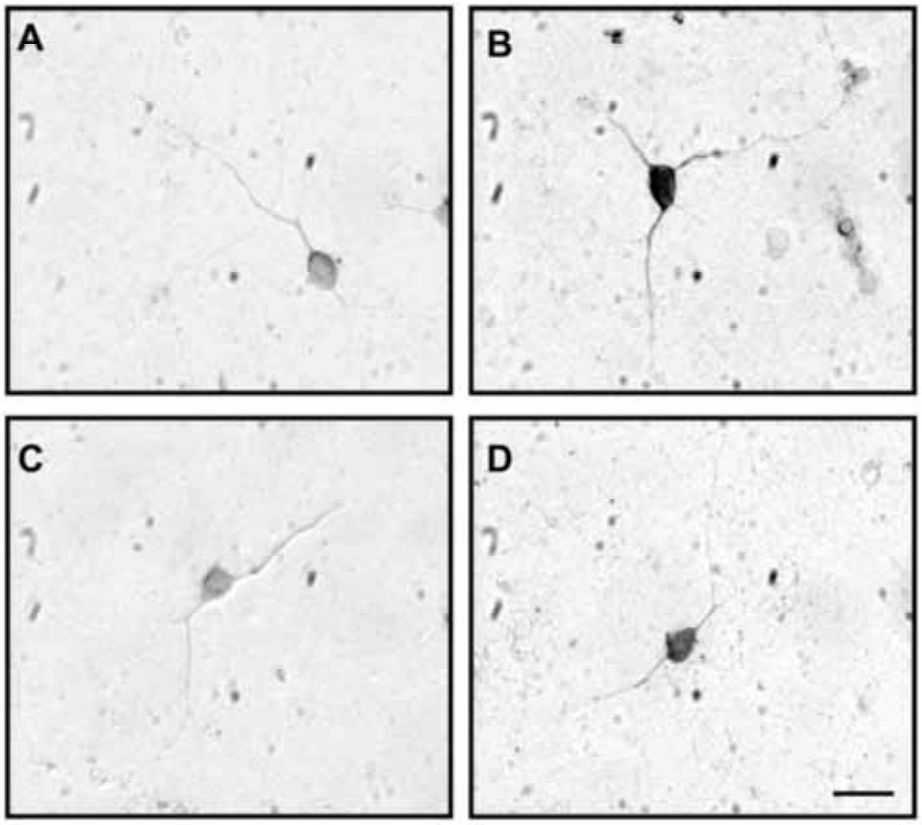
Striatal neuron development, maturation and establishment of synaptic connections are regulated by different neurotrophic factors (Maisonpierre et al., 1990; Checa et al., 2000; Ciccolini and Svendsen, 2001).



E **Quantitative analysis of the differentiation induced by GDNF on GABA-positive striatal neurons.**

	Total area (μm^2)	Perimeter (μm)	Soma area (μm^2)	Axon length (μm)	Degree of arborization
CONTROL	157 ± 4	177 ± 5	74 ± 4	44 ± 2	18 ± 2
GDNF	201 ± 6 *	246 ± 9 *	79 ± 5	61 ± 4 *	25 ± 1 *
GDNF+PD	158 ± 11	165 ± 16	79 ± 3	44 ± 3	14 ± 2
GDNF+LY	153 ± 5	172 ± 1	71 ± 2	44 ± 2	16 ± 0

Fig. 5. GDNF promotes GABA-positive neurons differentiation through the activation of the p42/p44 MAPK pathway. GABA immunocytochemistry was carried out at 7DIV. Photomicrographs show GABA-positive neurons from striatal cultures treated with either (**A**) vehicle, (**B**) GDNF (50 ng/ml), (**C**) GDNF plus PD98059 (50 μM) or (**D**) GDNF plus LY294002 (25 μM). Scale bar = 40 μm. **E:** Quantitative analysis of the effects of GDNF and the specific inhibitors on the morphology of striatal GABA-positive neurons. For each parameter and condition examined, 60 neurons were analyzed in three different experiments. Results are expressed as the mean ± SEM. *$P < 0.01$ compared to control values. ANOVA followed by the L.S.D. post-hoc test.



E **Quantitative analysis of the differentiation induced by GDNF on calbindin-positive striatal neurons.**

	Total area (μm^2)	Perimeter (μm)	Soma area (μm^2)	Axon length (μm)	Degree of arborization
CONTROL	193 ± 6	213 ± 7	89 ± 3	51 ± 3	19 ± 1
GDNF	266 ± 13 *	328 ± 16 *	94 ± 2	73 ± 5*	33 ± 2 *
GDNF+PD	194 ± 11	218 ± 14	88 ± 3	53 ± 4	21 ± 2
GDNF+LY	196 ± 15	204 ± 16	92 ± 6	55 ± 7	19 ± 1

Fig. 6. GDNF promotes the arborization of striatal calbindin-positive neurons through the activation of the p42/p44 MAPK pathway. Calbindin immunocytochemistry was carried out at 7DIV. Photomicrographs show calbindin-positive neurons from striatal cultures treated with either (**A**) vehicle, (**B**) GDNF (50 ng/ml), (**C**) GDNF plus PD98059 (50 μM) or (**D**) GDNF plus LY294002 (25 μM). Scale bar = 40 μm. **E:** Quantitative analysis of the effects of GDNF and the specific inhibitors on the morphology of striatal calbindin-positive neurons. For each parameter and condition examined, 60 neurons were analyzed in three different experiments. Results are expressed as the mean ± SEM. $^*P < 0.001$ compared to control values. ANOVA followed by the L.S.D. post-hoc test.

GDNF expression in the striatum varies during postnatal development with two peaks of expression on postnatal days (PND) 2 and 14 (Oo et al., 2005). This striatal GDNF has been mainly related with the survival of nigrostriatal dopaminergic neurons through a target-derived neurotrophic mechanism (Oo et al., 2003; Kholodilov et al., 2004). We show, however, that GDNF also regulates one aspect of striatal neuron development, the extension of neurites which occurs late in postnatal development. Accordingly, GFRα1 is expressed by striatal projection neurons which maximal levels between PND 10 and 14 (Cho et al., 2004). Furthermore, our results show that GDNF-mediated effects were higher on mature striatal neurons, the calbindin-positive population, and that these effects were mediated by the activation of the p42/p44 MAPK pathway. Similarly, previous studies have related the activation of this pathway in the striatum with the regulation of mature neuronal functions such behavioral plasticity and drug addiction (Mazzucchelli et al., 2002).

In conclusion, our data demonstrate that GDNF, through the activation of the p42/p44 MAPK pathway, specifically promotes striatal neuron differentiation more strongly in the calbindin-positive population. This indicates that GDNF plays a main role in inducing late stages of striatal neuron maturation. Furthermore, GDNF-mediated effects require a basal activity of the PI3-K pathway.

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SEGUNDO TRABAJO: *"Bax Deficiency Promotes a Differential Up-regulation of Bim_{EL} and Bak During Striatal and Cortical Postnatal Development, and After Excitotoxic Injury".*

(Enviado para su revisión)

OBJETIVO 2.- Estudio de la regulación de las proteínas de la familia de Bcl-2 en los procesos de muerte neuronal en el núcleo estriado.

2.1.- Estudio de una posible compensación por parte de las proteínas de la familia de Bcl-2 en animales deficientes para Bax durante el desarrollo postnatal del núcleo estriado y la corteza cerebral.

2.2.- Análisis de la regulación de las proteínas de la familia de Bcl-2 en respuesta a una lesión excitotóxica en ratones control y KO para Bax.

Las proteínas de la familia de Bcl-2 tienen un papel esencial en la regulación de diferentes procesos de muerte neuronal, además, trabajos previos realizados en nuestro laboratorio han demostrado un papel fundamental de estas proteínas en la muerte de las neuronas estriatales en un modelo excitotóxico de la enfermedad de Huntington. Concretamente se había observado que la proteína Bax tiene un papel esencial en la muerte neuronal que se produce en este modelo. Así, para abordar los puntos 2.1. y 2.2. del segundo objetivo, decidimos estudiar la regulación de las proteínas de esta familia y su participación en el proceso apoptótico en dos paradigmas experimentales diferentes: (1) durante el desarrollo postnatal del núcleo estriado y de la corteza cerebral; y (2) en una lesión estriatal aguda modelo de enfermedad de Huntington, utilizando animales deficientes para Bax.

Bax deficiency promotes an up-regulation of Bim_{EL} and Bak during striatal and cortical postnatal development, and after excitotoxic injury

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Running title: Compensatory changes of Bcl-2 family proteins in Bax KO

Keywords: apoptosis, basal ganglia, Bcl-2, BH-3 only proteins, knockout

Abbreviations: CNS, central nervous system; cyt *c*, cytochrome *c*; KO, knockout; PBS, phosphate buffered saline; QUIN, quinolinate

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Abstract

Targeted disruption of the pro-apoptotic protein Bax produces limited phenotypic abnormalities during central nervous system development, but prevents excitotoxic-induced cell death in the striatum. Here, we examined the compensatory mechanisms activated by the lack of Bax during striatal and cortical postnatal development, and after striatal excitotoxic lesion. Compared with wild-type animals, Bax knockout mice showed increased levels of the BH3-only protein Bim_{EL} in both brain areas but with a distinct temporal pattern. Furthermore, Bak was increased but only in the cortex at early stages of development, whereas Bad, Bcl-2 and Bcl-x_L were not modified. Excitotoxic-induced injury in the adult striatum increased Bim_{EL} in both wild-type and Bax knockout mice whereas Bak and Bcl-x_L were only increased in the latter. However, a translocation of Bim_{EL} protein from the cytosol fraction to the mitochondrial fraction was only observed in wild-type striata. Moreover, excitotoxicity only induced cytochrome *c* release in wild-type striata, presumably related to a decrease in Bax:Bcl-2 heterodimer levels and increased levels of Bax in the mitochondrial fraction. In contrast, Bak:Bcl-x_L heterodimers were not modified after lesion. In conclusion, our results show that in Bax deficient mice, other members of the Bcl-2 family, such as Bim_{EL} and Bak, are specifically regulated during postnatal development, suggesting that these proteins could compensate for the Bax function. In contrast, Bax is required to induce apoptosis after excitotoxicity in the adult striatum.

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.[1] Apoptosis can be activated through the extrinsic and intrinsic cell death pathways. Death signals activating the intrinsic pathway affect mitochondrial function, leading to the release of apoptosis-regulatory factors from mitochondria, such as cytochrome *c* (cyt *c*) or Smac/Diablo which participate in the initiation of the caspase cascade in the cytosol. One of the major regulators of the mitochondrial integrity is the Bcl-2 family,[2] which comprises both pro-apoptotic and anti-apoptotic proteins. Pro-apoptotic proteins cause

mitochondrial dysfunction leading to the release of apoptogenic factors, while the anti-apoptotic members of the Bcl-2 family prevent these events.

Proteins belonging to the Bcl-2 family share, at least, one conserved region termed Bcl-2 homology (BH) which mediates protein-protein interactions. Anti-apoptotic members, such as Bcl-x_L, Bcl-w, Mcl-1 and Bcl-2, contain four BH (BH1-4) domains whereas the pro-apoptotic members of this family are divided into two subgroups: multidomain proteins that contain BH1-3 domains, such as Bax and Bak, and BH3-only members, such as Bad, Bid, Hrk and Bim.[3] The intracellular balance between members of the Bcl-2 family and their interactions regulates the activation of apoptosis after death stimuli. Thus, for example, Bcl-2 and Bcl-x_L can inhibit Bax through heterodimerization,[4,5] while BH3-only proteins can bind to anti-apoptotic proteins suppressing their pro-survival function, or can directly activate pro-apoptotic proteins Bax and Bak.[6,7] The function of Bcl-2 family members can also be modified at the transcriptional level.[3] In fact, increased expression of Bax[8,9,10] or Bim[11,12,13] and reduced levels of Bcl-2 and/or Bcl-x_L[8,9,10] have been detected in response to several injuries to the nervous system.

Consistent with the role of Bax in the execution of apoptosis after insults to the nervous system, Bax knockout (KO) mice are less sensitive to various cell death stimuli. Bax KO cultured sympathetic neurons and motoneurons show resistance to trophic factor withdrawal,[14] cerebellar neurons to potassium deprivation[15] and cortical cells to glutamate-induced excitotoxicity.[16] Furthermore, Bax KO mice show less dopaminergic cell death after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine administration,[17] resistance to ischemia-mediating neuronal loss in the hippocampus[18] and a reduction in striatal cell death induced by excitotoxicity.[10] Although all these studies show the involvement of Bax in stimulus-induced cell death, its role in naturally occurring cell death seems specific to some neuron populations and developmental stages[14,19,20], suggesting that other Bcl-2 family members may participate in this process. Therefore, the aim of the current study was to determine whether differences

in expression of other members of the Bcl-2 family (Bak, Bim, Bad, Bcl-2 and Bcl-x_L) can compensate for Bax deficiency and contribute to the control of postnatal development. To assess whether compensatory changes to the absence of Bax in the brain are a general mechanism, we focused on the study of two regions, the striatum and cortex, which participate in motor control and degenerate in Huntington's disease.[21] We also examined the consequences triggered by Bax deficiency in members of the Bcl-2 family in response to an excitotoxic lesion in the striatum.

Results

Bax KO mice do not show striatal or cortical abnormalities

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 the highest levels at P3 and P7, and then falling from P15 until adulthood (Fig. 1).

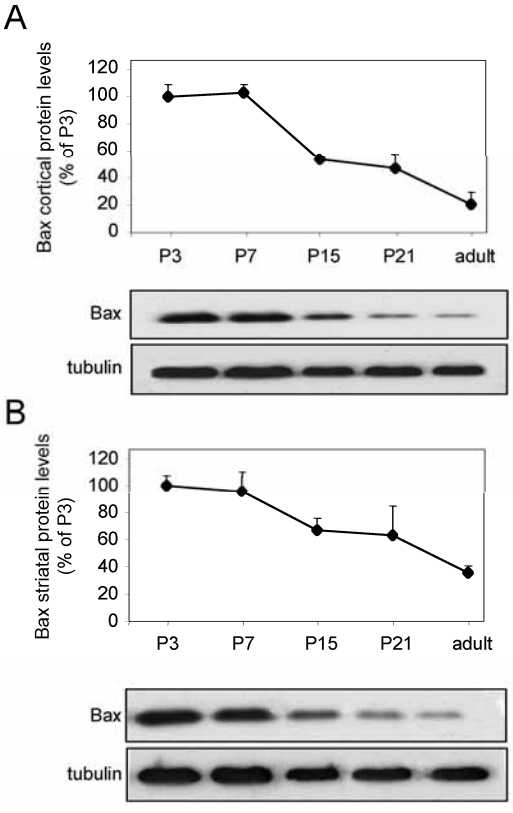

Figure 1.- Bax follows a similar pattern of expression during cortical and striatal postnatal development. The expression levels of Bax were examined by Western blot in the cortex (a) and striatum (b) at different postnatal ages. -tubulin was used as loading control. Results obtained from densitometric measures represent the ratio between Bax and -tubulin levels, and are expressed as percentages of P3 values ± SEM for five animals per condition. Immunoblots were obtained from representative experiments.

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 (8 week-old). No significant differences were observed in the density of NeuN-positive cells (cells/mm^2) between wild-type and Bax KO animals in any of the regions examined (Striatum: wild-type, 83340 ± 7877; KO, 86280 ± 5240; Cortex: wild-type, 53902 ± 7177; KO, 48697 ± 1909). Furthermore, striatal volume (wild-type: 10.8 ± 0.2 mm^3; KO: 11.1 ± 0.2 mm^3) and the total striatal cell number (wild-type: 896876 ± 81825; KO: 961155 ± 75500) were similar in both genotypes.

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 for Bax deficiency. We therefore examined protein levels of Bak, Bim, Bad, Bcl-2 and Bcl-x_L during the postnatal development of wild-type and Bax KO mice.

Bak did not follow the same pattern of expression as Bax in the cortex and striatum during postnatal development. In the cortex of wild-type animals the highest Bak levels were observed at P7 and P21 (Fig. 2a) while in the striatum Bak levels increased from P3 to P7, remained stabled until P21 and then decreased dramatically to reach adult levels (Fig 2b). In Bax KO mice, protein levels were modified compared to wild-type animals, but only in the cortex of P3 mice, where we observed an increase of 67 ± 8% (Fig. 2a).

In mice, three major Bim isoforms generated by alternative splicing have been identified and designated as Bim_{EL}, Bim_L and Bim_s.[22] Among these isoforms, we only detected Bim_{EL} in all the regions and at all ages analyzed, in agreement with previous data showing that in the central nervous system (CNS) Bim_{EL} expression is dominant compared to other isoforms in terms of both intensity and distribution.[23] In wild-type animals Bim_{EL} expression showed a

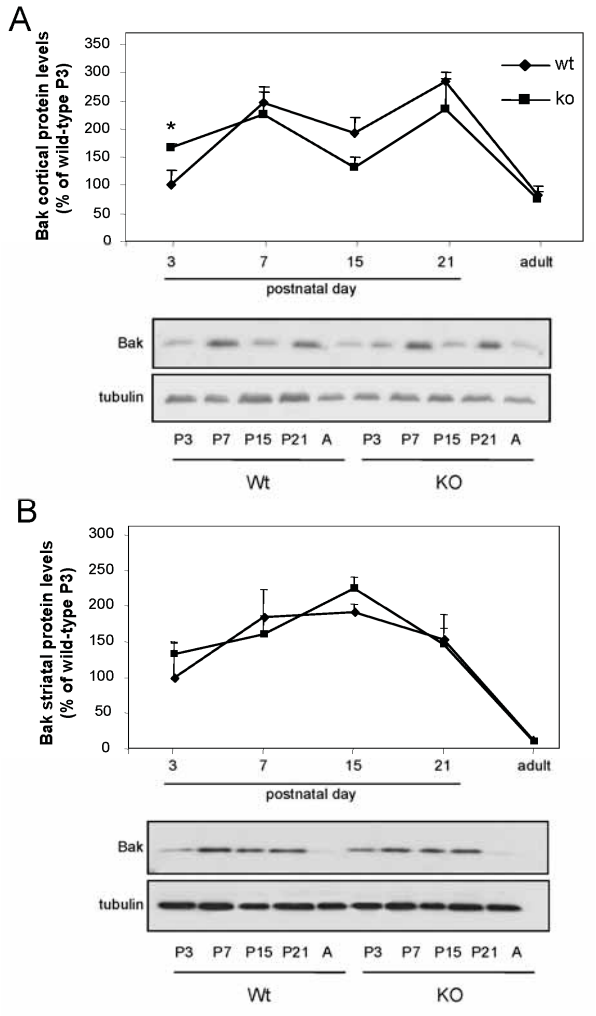

Figure 2.- Bak is differentially expressed, and regulated in the absence of Bax, in cortex and striatum during postnatal development. Cortical (a) and striatal (b) Bak protein levels were analyzed by Western blot in wild-type (diamond; Wt) and Bax KO (square; KO) mice at different postnatal ages. - tubulin was used as loading control. Results obtained from the analysis of Western blots were normalized to - tubulin protein levels and expressed as percentages of P3 wild-type values ± 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 (two-way ANOVA followed by Scheffe´s post hoc test). Immunoblots show representative experiments.

different pattern during cortical and striatal postnatal development (Fig. 3a and b). In the cortex, its levels of expression increased slightly at P7, were similar to P3 levels between P15 and P21 and then fell to adult levels (Fig. 3a). In the striatum, the maximum levels were detected at P3, decreasing until P15 when adult levels were reached (Fig. 3b). When compared to wild-type animals, Bax KO mice showed increased Bim_{EL} protein levels in both brain areas but at different postnatal ages (Fig. 3a and b). 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_{EL} protein levels were only modified at later postnatal ages (P21 and adulthood; Fig. 3b).

Analysis of Bad expression also found differences between cortex and striatum (Fig. 3c and d). In wild-type animals, cortical Bad protein levels increased progressively

over time, reaching a peak in adulthood (Fig. 3c). In contrast, striatal Bad protein expression increased slightly at P7 with maximum levels detected at P21 (Fig. 3c).

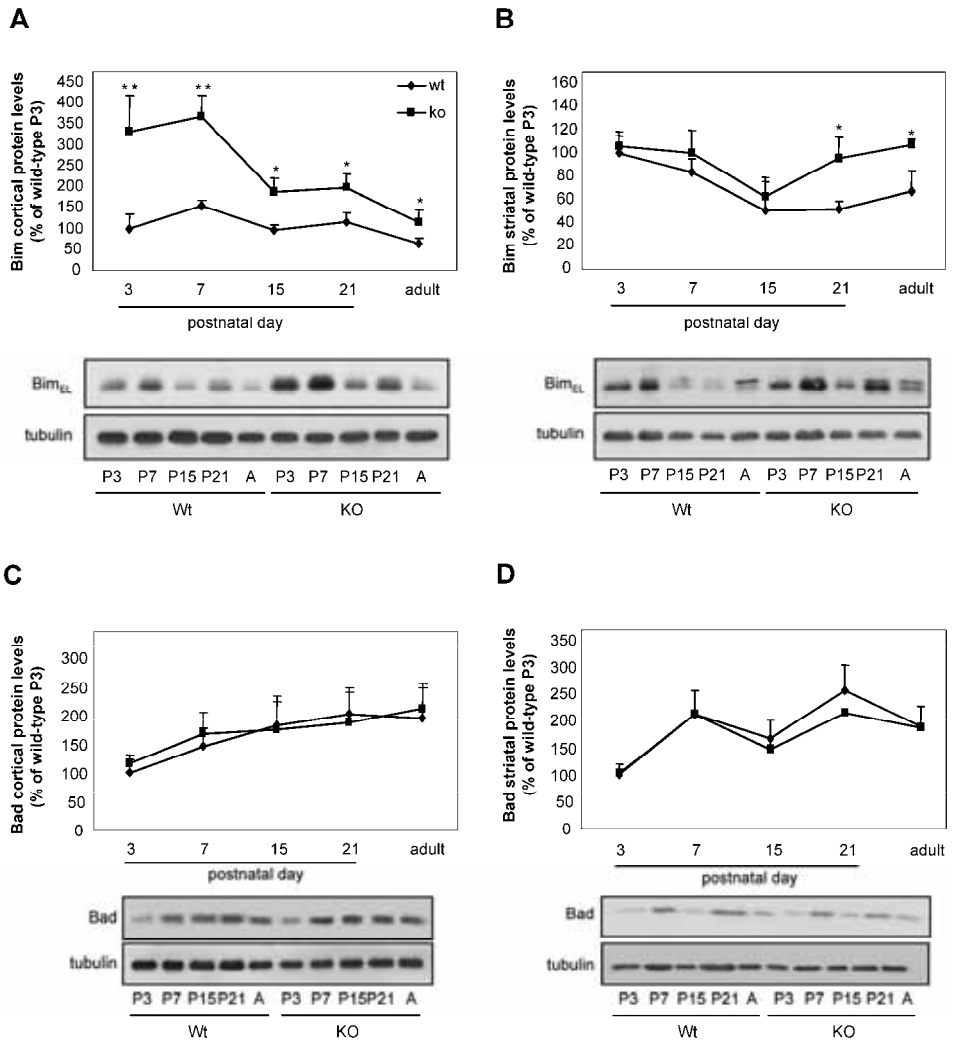

Figure 3.- Differential expression and regulation of BH3-only proteins, Bim$_{EL}$ and Bad, in the cortex and striatum during postnatal development of wild-type and Bax KO animals. Cortical (a and c) and striatal (b and d) protein levels of Bim$_{EL}$ (a and b) and Bad (a and d) were analyzed by Western blot at different postnatal ages in wild-type (diamond, Wt) and Bax KO (squares, KO) mice. Results were normalized to levels of the loading control -tubulin and expressed as percentages of protein levels in P3 wild-type mice ± SEM for five animals per condition. Increased levels of Bim$_{EL}$ 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. In contrast Bad protein levels were similar in striatum and cortex of wild-type and Bax KO mice at all postnatal ages analyzed. $^{**}p < 0.01$, $^{*}p < 0.05$ compared with corresponding values in wild-type animals (two-way ANOVA followed by the Scheffe´s post hoc test). Immunoblots were obtained from representative experiments.

No differences between wild-type and Bax KO animals were observed in Bad expression during postnatal development in any of the brain regions examined (Fig. 3c and d).

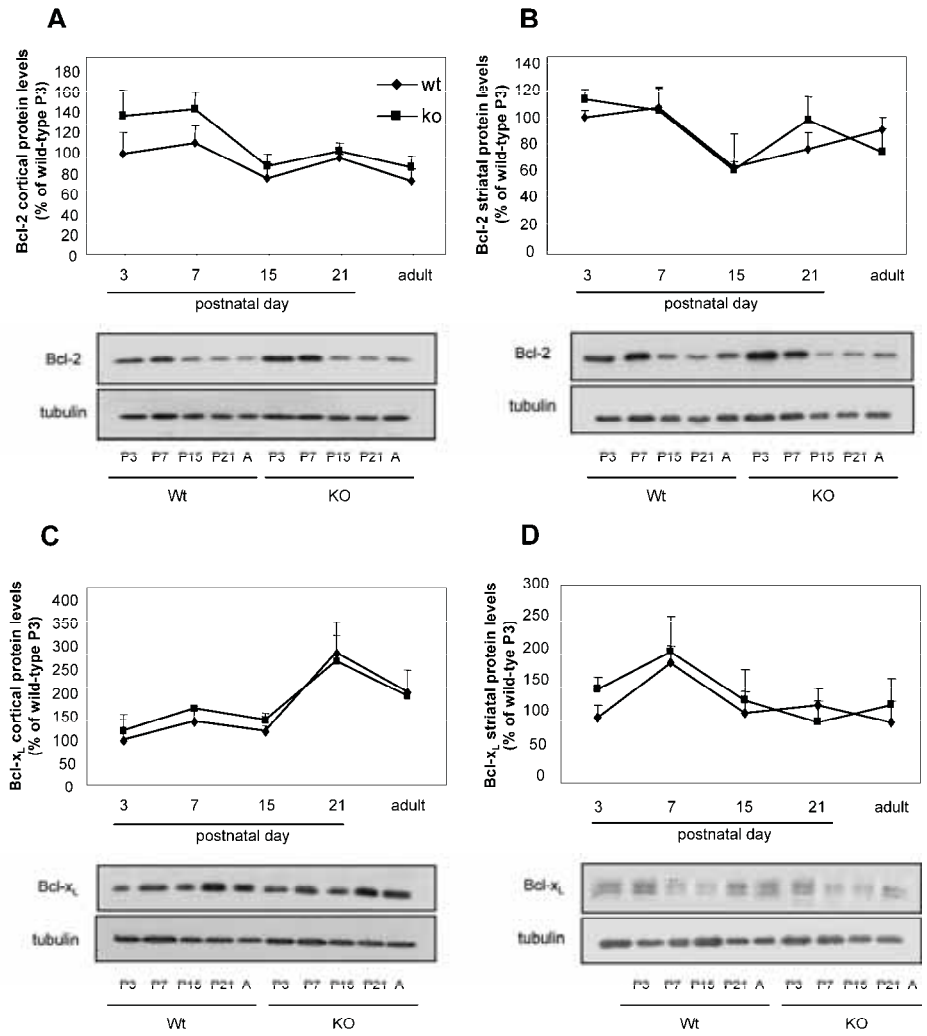

Figure 4.- Bax deficiency does not affect the expression of Bcl-2 and Bcl-x_L proteins during postnatal development. Protein levels of Bcl-2 (a and b) and Bcl-x_L (c and d) were analyzed by Western blot at different postnatal ages in the cortex (a and c) and striatum (b and d) of wild-type (diamonds, Wt) and Bax KO mice (squares, KO). Results obtained from the analysis of Western blots were normalized to -tubulin protein levels and expressed as percentages of P3 wild-type values ± SEM for five animals per condition. Immunoblots were obtained from representative experiments.

Finally, pro-survival proteins Bcl-2 and Bcl-x_L were examined (Fig. 4). 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 to levels that were maintained until adulthood (Fig. 4a and b). 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. 4c) whereas in the striatum maximum levels were reached at P7 (Fig. 4d). In Bax KO mice, the pattern of expression of Bcl-2 and Bcl-x_L in the cortex and striatum was similar to that in wild-type animals (Fig. 4c and 4d).

Excitotoxicity differentially regulates the expression of Bcl-2 family members in the striatum of wild-type and Bax KO animals

In order to examine the regulation of Bcl-2 family members by excitotoxicity, quinolinate (QUIN, an NMDA glutamate receptor agonist) was injected in the striatum of wild-type and Bax KO mice at 8 weeks of age. First, we analyzed cell death in wild-type animals by the TUNEL assay at 24, 48 and 72 h after lesion. Several positive nuclei were detected at 24 h (Fig. 5a) which increased at 48 h (Fig. 5b) and 72 h post-lesion (Fig. 5c). We therefore decided to perform all the analyses at 48 h after QUIN intrastriatal injection.

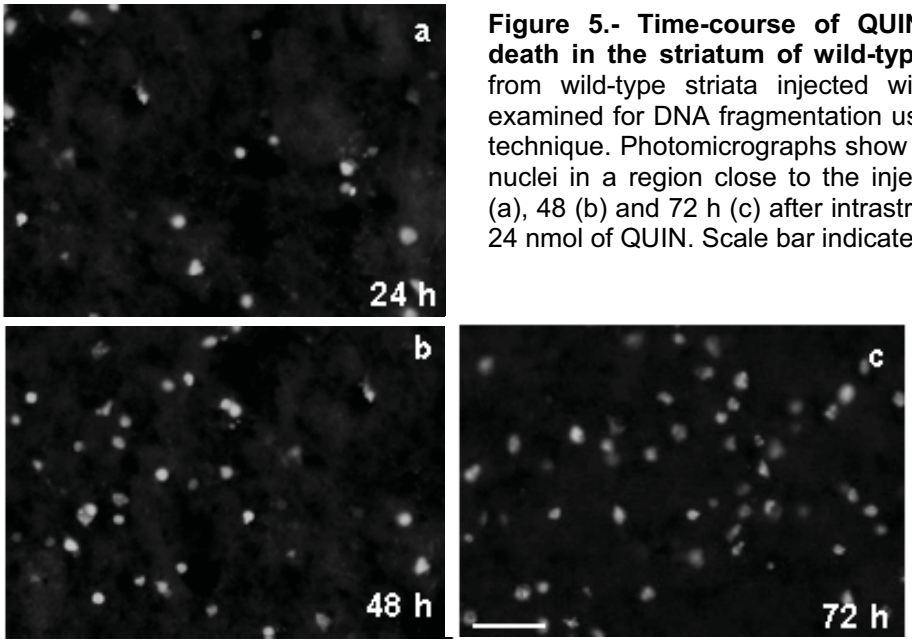

Figure 5.- Time-course of QUIN-induced cell death in the striatum of wild-type mice. Striata from wild-type striata injected with QUIN were examined for DNA fragmentation using the TUNEL technique. Photomicrographs show TUNEL-labeled nuclei in a region close to the injection site at 24 (a), 48 (b) and 72 h (c) after intrastriatal injection of 24 nmol of QUIN. Scale bar indicates μm.

QUIN injection in wild-type striatum did not modify Bax protein levels (93 ± 16% of non-injected striatum). In contrast, although Bim_{EL} protein levels were already higher in non-injected Bax KO than in wild-type striata, excitotoxicity caused a similar regulation of this protein in both genotypes (Fig. 6). An increase was observed 48 h after QUIN injection in wild-type (by 80 ± 32%; $p < 0.05$) and in Bax KO striata (by 47 ± 11%; $p < 0.05$). In contrast, protein levels of Bak (by 105 ± 25%; $p < 0.01$) and Bcl-x_L (by 36 ± 10%; $p < 0.05$) were only up-regulated in Bax KO animals (Fig. 6). Moreover, neither Bcl-2 (wild-type: 92 ± 4%, Bax KO: 106 ± 9%) nor Bad (wild-type: 86 ± 6%, Bax KO: 93 ± 9%) protein levels were modified by intrastriatal QUIN injection.

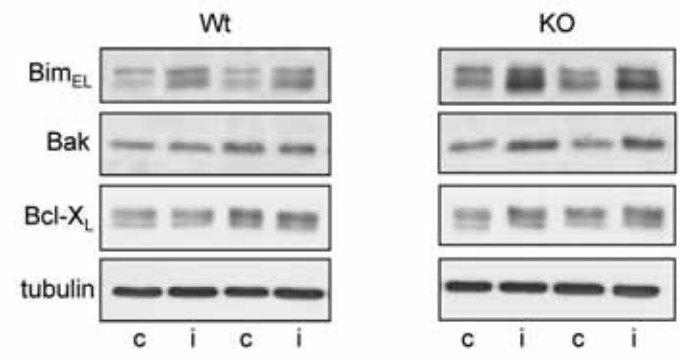

Figure 6.- Bcl-2 family members are differentially regulated in wild-type and Bax KO animals by intrastriatal QUIN injection. BimEL, Bak and Bcl-x_L were analyzed by Western blot 48 h after QUIN injection in wild-type (Wt) and Bax KO (KO) striata. Bim$_{EL}$ protein levels were similarly increased by intrastriatal QUIN injection in wild-type and Bax KO animals whereas the levels of Bak and Bcl-x_L were only increased in Bax KO mice. Immunoblots show results obtained in two different animals. Protein levels were normalized with the corresponding signal of -tubulin. c: striatum contralateral to the lesion; i: striatum ipsilateral to the lesion.

Bim does not form heterodimers with Bcl-2, Bax or Bak

The increased levels of Bim$_{EL}$ indicated that this BH3-only protein was possibly involved in the initiation of the apoptosis after the excitotoxic insult. We therefore analyzed whether the Bim$_{EL}$ protein was acting by displacing Bcl-2 from Bax or Bak, or

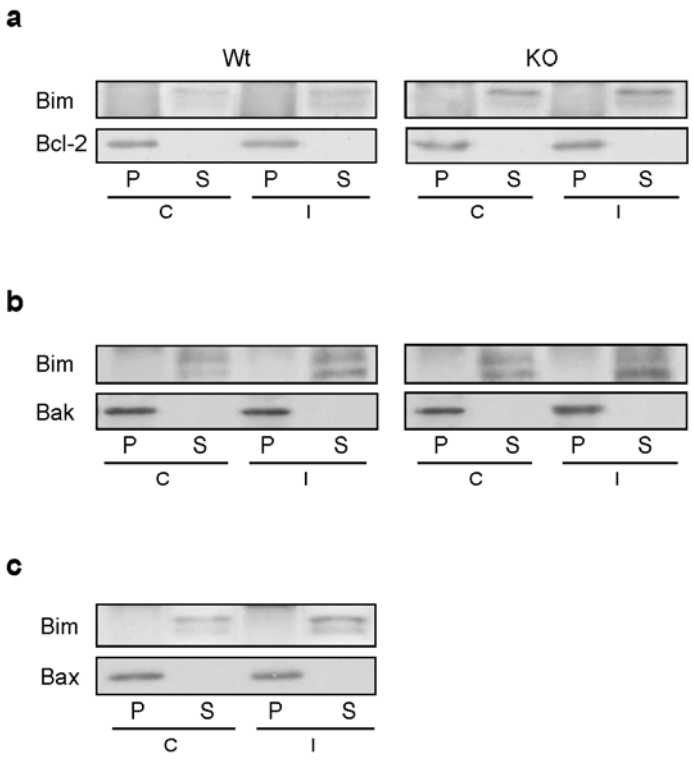

Figure 7.- Bcl-2, Bax and Bak did not interact with Bim$_{EL}$. (a) Bcl-2:Bim$_{EL}$ and (b) Bak:Bim$_{EL}$ heterodimers were analyzed by immunoprecipitation in the striatum of wild-type (Wt) and Bax KO (KO) mice without lesion (c: striatum contralateral to the lesion) and 48 h after QUIN injection (i: striatum ipsilateral to the lesion). (c) Bax:Bim heterodimerization was also examined by immunoprecipitation in non-injected (C) and in QUIN-lesioned (I) wild-type striatum (Wt). Immunoblots were obtained from representative experiments. P, pellet; S, supernatant.

interacting directly with the multidomain pro-apoptotic proteins. Immunoprecipitation analysis showed that Bim_{EL} did not form heterodimers with Bcl-2 in any of the conditions examined (wild-type and Bax KO striata non-injected or injected with QUIN; Fig. 7a). The study of Bim_{EL} association with Bak in the striatum of non-injected and QUIN-lesioned wild-type and Bax KO striata showed that this protein did not co-immunoprecipitate with Bak (Fig. 7b). Furthermore, Bim_{EL} also failed to interact with Bax in all the conditions examined (Fig. 7c).

QUIN injection induces relocalization of pro-apoptotic proteins and cyt *c* release in wild-type but not in Bax KO striata

To determine whether changes in Bim_{EL} protein levels were accompanied by changes in its subcellular localization we examined protein levels in the mitochondrial fraction extracted from QUIN-injected wild-type and Bax KO striata. In non-injected Bax KO striata, Bim_{EL} levels in the mitochondrial fraction were higher than those in wild-type animals, and were not modified by excitotoxicity (Fig. 8a). In contrast, QUIN injection in wild-type striata induced an increase in the content of Bim_{EL} protein in the mitochondrial fraction (Fig. 8a). Furthermore, wild-type striata also showed increased levels of Bax protein in the mitochondrial fraction 48 h after QUIN injection (Fig. 8b). We next analyzed whether changes in the subcellular localization of Bax and Bim_{EL} were able to induce the release of pro-apoptotic factors from the mitochondria to the cytosol. To this end, cyt *c* and Smac/Diablo protein levels were determined by Western blot in cytosolic fractions obtained from QUIN-injected striata. No significant differences in the levels of Smac/Diablo were observed in any of the conditions examined (data not shown). In contrast, cyt *c* was highly increased in cytosolic fractions obtained from QUIN-injected wild-type (by 346 ± 70%; $p < 0.001$), but not Bax KO, striata (Fig. 8c).

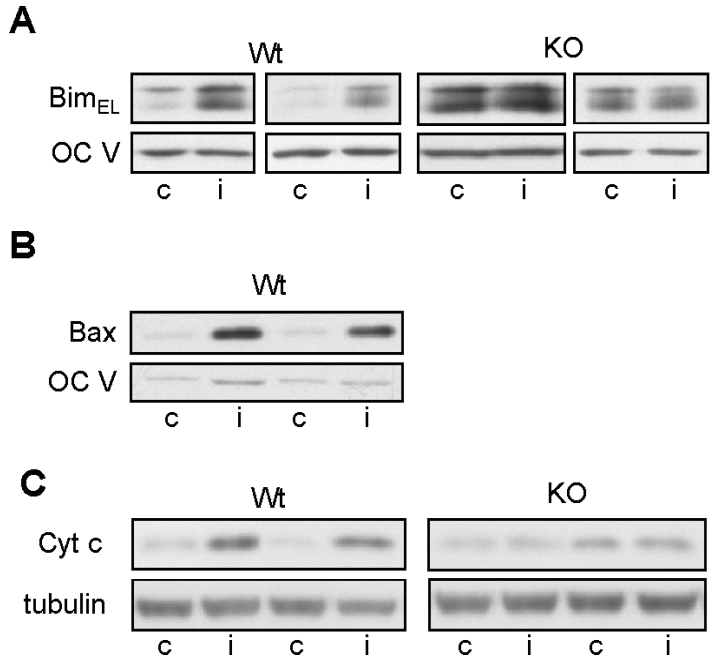

Figure 8.- Cyt *c* release is induced by intrastriatal QUIN injection in wild-type but not in Bax KO mice. Subcellular fractionation analysis of Bim_{EL} (a), Bax (b) and Cyt *c* (c) 48 h after lesion. Mitochondrial fractions obtained from wild-type striata (Wt) injected with QUIN underwent Western blot using antibodies to Bim_{EL} (a) and Bax (b) while those obtained from Bax KO (KO) injected striata were analyzed for Bim_{EL} protein levels (b). Cyt *c* levels were analyzed in cytoplasmic fractions from QUIN-injected wild-type (c, Wt) and Bax KO (c, KO) striata. Oxidative Complex V (OC V) and -tubulin were used as loading controls for mitochondrial and cytosolic fractions, respectively. Immunoblots show results obtained in two different animals. C: striatum contralateral to the lesion; I: striatum ipsilateral to the lesion.

Bax:Bcl-2 but not Bak:Bcl-x_L heterodimer levels are modified by QUIN injection

Although pro-apoptotic proteins Bak and Bim_{EL} were enhanced by QUIN injection in Bax KO striatum, we did not detect cyt *c* release. Since the pro-apoptotic properties of Bak can be regulated through heterodimerization with pro-survival proteins, we assessed the capacity of Bcl-2 and Bcl-x_L to heterodimerize with Bak in non-lesioned and in QUIN-injected wild-type and Bax KO striata. As shown in Figure 9a, Bak did not form heterodimers with Bcl-2 in any of the conditions examined. However, Bak can be heterodimerized with Bcl-x_L in non-lesioned wild-type and Bax KO striata, and this dimerization was not disrupted by the excitotoxic stimulus (Fig. 9a). In contrast, QUIN injection in wild-type striata reduced the amount of Bcl-2 that co-immunoprecipitated with Bax (Fig. 9b) suggesting that Bax, freed from Bcl-2, may translocate to the mitochondria participating in the release of cyt *c*.

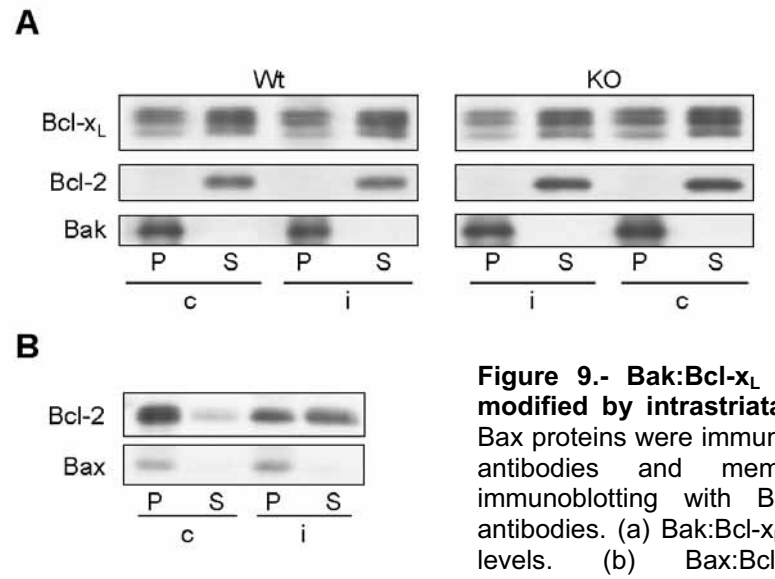

Figure 9.- Bak:Bcl-x_L heterodimerization is not modified by intrastriatal QUIN injection. Bak and Bax proteins were immunoprecipitated with polyclonal antibodies and membranes then underwent immunoblotting with Bcl-x_L, Bcl-2, Bak or Bax antibodies. (a) Bak:Bcl-x_L and Bak:Bcl-2 heterodimer levels. (b) Bax:Bcl-2 heterodimer levels. Heterodimerization was examined in non-lesioned (C: striatum contralateral to the lesion) and in QUIN-lesioned (I: striatum ipsilateral to the lesion) wild-type (Wt) and Bax KO (KO) striata. Immunoblots were obtained from representative experiments. P, pellet; S, supernatant.

Discussion

Our data show that the regulation of Bax protein levels during the postnatal development of the cortex and striatum follows a similar pattern of expression. However, the lack of Bax does not modify the number of adult neurons in these two brain areas, indicating the existence of alternative or redundant pathways that can control cell death during this developmental period. Here, we report that among all the Bcl-2 family members analyzed, only the pro-apoptotic proteins Bim_{EL} and Bak are regulated by the absence of Bax in a brain region- and developmental stage-dependent manner. Our findings also show that excitotoxicity differentially regulates Bcl-2 family proteins in the striatum of wild-type and Bax null mutant mice. Furthermore, although in Bax KO mice striatum other pro-apoptotic proteins such as Bim_{EL} and Bak are up-regulated by excitotoxicity, cell death is reduced,[10] suggesting that Bax is necessary to execute cell death under these circumstances.

In agreement with previous data[24] our results show that the death-promoting protein Bax was highly expressed in the cortex and striatum between P3 and P7, correlating with the cell death that takes place during these developmental stages[25,26]

and suggesting that Bax may participate in the regulation of these apoptotic processes. However, the stereological analysis of the number of cortical and striatal neurons in adult Bax KO mice showed no differences compared with wild-type animals, indicating that other alternative pathways may exist. Thus, Bax involvement in developmental cell death might depend on the neuronal type considered, since other populations of the peripheral and CNS are altered in Bax KO mice.[19,20] However, the other three pro-apoptotic proteins analyzed, Bak, Bim and Bad showed different patterns of expression in the cortex and striatum, suggesting that the relative importance of each member of the Bcl-2 family may vary in particular brain regions. In agreement with this specificity, it has been shown that mitochondria of striatal and cortical neurons show differential sensitivity to calcium-induced permeability transition[27] and they differentially activate Bcl-2 family members in response to metabolic compromise.[28]

The analysis of pro-survival proteins showed that Bcl-2 followed a similar expression pattern in cortex and striatum, with a decrease at P15 that was maintained until the adulthood. In contrast, the highest levels of Bcl-x_L in cortex and striatum were detected at different postnatal ages, although in both regions the levels of expression of this protein were similar in P3 and adult mice. Our results are in accordance with those showing different developmental patterns of expression of Bcl-2 and Bcl-x_L in the CNS with a reduction of Bcl-2 levels with age[29,30] and maintenance of Bcl-x_L expression through adulthood.[31,32] Moreover, the present data suggest that the maintained Bcl-x_L expression may participate in neuronal survival when Bcl-2 levels decline.

Our results indicate that there is a fine balance between pro- and anti-apoptotic Bcl-2 members, specific for each region and developmental stage, which may participate in the regulation of cell death. This balance could be modified in the absence of any of the proteins examined. To test this hypothesis we examined Bcl-2 family members in Bax null mutant mice in the same regions and postnatal ages as in the wild-type mice. The results obtained showed no changes in Bcl-2, Bcl-x_L and Bad protein levels, while pro-apoptotic proteins Bak and Bim_{EL} were specifically regulated,

suggesting that these proteins could compensate for Bax deficiency. The most striking feature observed was that Bax KO mice displayed increased levels of Bim_{EL} in both brain areas examined, although at different postnatal ages. It has been shown that Bim is one of the most potent BH3-only proteins in inducing apoptosis because it antagonizes all the pro-survival proteins[33] and can also directly activate the Bax pro-apoptotic function.[6,34] Furthermore, it has been suggested that induction of Bim_{EL} is critical for neuronal apoptosis.[35] Therefore, our results suggest that in the absence of Bax, cortical and striatal neurons respond by up-regulating Bim_{EL} protein levels to ensure cell death at specific periods.

The results obtained during postnatal development showed that striatal neurons from both wild-type and Bax KO mice responded similarly to excitotoxicity by up-regulating Bim_{EL} protein levels. Therefore, the excitotoxic stimulus induced this protein in a Bax-independent manner, indicating that Bim_{EL} acts upstream of Bax as has been shown in cerebellar neurons from both wild-type and Bax KO mice after potassium deprivation.[36] Other *in vitro* studies also show the involvement of Bim_{EL} in neuronal death induced by potassium deprivation,[35] trophic factor withdrawal[35,37] or hyperglycemia.[38] Furthermore, Bim_{EL} is also involved in neuronal apoptosis *in vivo* as in hippocampal cell death induced by seizures,[12] in ischemia-induced cortical and striatal cell death[13,39] and in retinal ganglion cells after optic nerve transection.[11] Taken together, these results show that the enhancement of Bim_{EL} protein levels is a common mechanism activated in neurons in response to harmful stimuli, suggesting that levels of this BH3-only protein may set the threshold for initiation of neuronal apoptosis.

The exact mechanism by which the BH3-only proteins activate apoptosis is not clearly defined. It has been proposed that BH3-only proteins can sequester anti-apoptotic proteins such as Bcl-2 and Bcl-x_L allowing the release of previously inhibited proteins Bax and Bak, or can directly activate multidomain pro-apoptotic proteins.[7] Our data indicate that Bim_{EL} acts via a mechanism other than displacement of pro-survival proteins from Bax or Bak, since we did not detect Bcl-2:Bim_{EL} heterodimerization after

excitotoxicity, and Bak was still sequestered by Bcl-x_L. Furthermore, after QUIN intrastriatal injection in wild-type, but not in Bax KO mice, we observed increased Bim_{EL} protein levels in the mitochondrial fraction. According to our results, induced Bim_{EL} localized in the mitochondrial membrane where it can contribute to the multimerization and integration of Bax into the mitochondrial outer membrane, facilitating cyt *c* release.[35,40] In fact, we also detected increased levels of Bax protein in the mitochondrial fraction and cyt *c* release after QUIN injection. Our results therefore suggest that Bim_{EL} could be activating Bax by a mechanism that is independent of their direct interaction, as we did not observe Bax:Bim_{EL} heterodimers after excitotoxicity.

Our data also indicate that the presence of Bax was necessary to execute cell death induced by excitotoxicity. Although high levels of Bim_{EL} were present in the mitochondrial fraction in adult non-lesioned Bax KO striatum, and Bak protein levels were increased by QUIN injection, cyt *c* release was not detected. Studies in Bax/Bak double mutant mice found that these two pro-apoptotic proteins have overlapping roles in the regulation of apoptosis[41,42,43] which may explain the increase in Bak protein levels in the Bax null mutant striatum in response to excitotoxicity. 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.[44] Moreover, it has been shown that Bak is sequestered by Bcl-x_L and Mcl-1, but not Bcl-2, and efficient killing occurs only if both Bcl-x_L and Mcl-1 are neutralized.[45] However, little is known about whether these interactions actually take place in neurons and *in vivo*. In this *in vivo* model we show that, like *in vitro*, Bak can interact with Bcl-x_L, but not with Bcl-2, and that this interaction occurs in the presence or absence of Bax. Furthermore, excitotoxicity did not disturb Bak:Bcl-x_L heterodimerization, which may explain the absence of cyt *c* in the striatum of Bax KO mice.

In summary, our results show that Bax deficiency induces a specific regulation of Bak and Bim_{EL} pro-apoptotic proteins during cortical and striatal postnatal development in a

regional and temporal manner, indicating that these proteins participate in the control of cell death pathways that are active during these postnatal periods. In response to excitotoxicity, wild-type and Bax KO striatal cells up-regulate Bim_{EL}, suggesting that this BH3-protein may act as a control point for striatal cell death. Furthermore, although Bax KO striatal cells respond to excitotoxicity by up-regulating Bak pro-apoptotic protein as well, cell death does not occur, which identifies Bax as a necessary protein for execution of cell death in response to excitotoxicity in striatal cells. Therefore, taken together, our results suggest that during cortical and striatal development Bax and Bak could have overlapping functions, whereas they play specific roles in injury-dependent striatal cell death.

Materials and Methods

Animal subjects

Heterozygous Bax (C57BL/6J; Jackson Laboratories, Bar Harbor ME, USA) mice were bred to maintain the colony and to obtain Bax -/-, +/-, and wild-type genotypes. Genotyping was performed as described elsewhere.[10] Male wild-type and Bax KO mice were sacrificed at different postnatal days (P; 3, 7, 15 and 21) and in adulthood (8 week-old) 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).

Immunohistochemistry

For immunohistochemical analysis adult wild-type and Bax KO animals (n = 5 for each condition) were deeply anesthetized and immediately perfused through the heart with PBS 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 μm) through the whole striatum were serially collected in PBS as free-

floating sections and stained with the NeuN (1:100; Chemicon, Temecula, CA, USA) antibody. After treatment with H_2O_2 (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 ^oC. After washing, they were incubated with biotinylated secondary antibodies (1:200) and then with the avidin-biotin complex (ABC kit, Pierce, Tattenhall, UK). Finally, sections were developed with 0.05% diaminobenzidine, 0.01% $NiCl_2$ and 0.02% H_2O_2. As negative immunohistochemical controls, some sections were processed as described above in the absence of primary antibody.

Cell counting

Stereological quantification was carried out using an optical dissector/Cavalieri combination. All cell counts were performed blind with respect to genotype in 8-week-old mice (n = 5 per each group). Unbiased stereological counts of striatal cells were obtained from the entire neostriatum and from 300 μm^2 of layer V of motor cortex using the Computer-Assisted Stereology Toolbox (CAST) software (Olympus). The dissector counting method was used to analyze coronal sections spaced 300 μm apart. The counting frames were randomly sampled.

Excitotoxic lesion

Wild-type and Bax KO mice (8 week-old) were anesthetized with pentobarbital and QUIN (24 nmol; Sigma Chemical Co., St Louis, MO, USA) was intrastriatally 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^oC) and humidity (40-50%) on a 12:12 hr light/dark cycle. For Western blot and Immunoprecipitation analysis, animals were killed by decapitation (n = 5 for each genotype) at 48 h post-lesion. Brains were rapidly removed and striata ipsilateral as well as contralateral to the lesion were dissected and frozen at -80ºC.

In situ detection of DNA fragmentation

Mice were deeply anaesthetized and immediately perfused transcardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde/phosphate buffer (0.1 M, pH 7.4) at 24, 48 and 72 h after intrastriatal QUIN injection (n = 4 for each genotype and time point). Brains were rapidly removed and post-fixed for 1-2 h in the same solution and cryoprotected by immersion in 15% sucrose/PBS. Brains were then frozen in dry ice-cooled isopentane and cryostat horizontal sections (14 μm) through the whole striatum were serially collected on silane-coated slides. DNA fragmentation was histologically examined using the in situ Apoptosis detection system, Fluorescein (Promega, Madison, WI, USA) and performed as described elsewhere.[46]

Subcellular fractionation

Mice were killed by decapitation (n = 7 for each genotype) at 48 h after QUIN injection. Brains were rapidly removed and striata ipsilateral as well as contralateral to the lesion were dissected and kept on ice until processed. Both cytosolic and mitochondrial fractions were extracted from fresh striatal samples as previously described.[47] Briefly, striatal tissue was gently homogenized in buffer A (250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 2 mM EDTA and 20 mM HEPES) plus protease inhibitors (2 mM phenylmethylsulphonyl fluoride (PMSF), 1 mg/μl aprotinin, 1 mg/μl leupeptin and 1 mM sodium orthovanadate) by passages through a 21Gx1 9/16" 0.8- by 40 mm and a 25Gx5/8" 0.5- by 16 mm needle fitted on a 2 ml syringe. The resulting homogenates were centrifuged at 500 g for 5 min at 4ºC. Supernatants (S1) were collected and centrifuged at 13000 g for 20 min at 4ºC. The resulting pellets (P2) were washed once in buffer A and centrifuged at 13000 g for 20 min at 4ºC to obtain the mitochondrial fraction (P3). The resulting supernatants (S2) were further centrifuged at 100000 g for 60 min at 4ºC. The supernatants (S3) were designated cytosolic fractions.

Western blot analysis.

Striatum and cortex were dissected out and homogenized in lysis buffer to obtain total protein extracts. Protein levels of Bcl-2 family members were analyzed by Western blot as described elsewhere.[10] Membranes were blotted with the following antibodies: Bax (1:1000) and Bad (1:1000) (Cell Signaling,

Beverly, MA, USA), Bcl-x_L (1:500) and Bcl-2 (1:2000) (Transduction Laboratories, Lexington, KY, USA), Bim (1:1000; Stressgen, San Diego, CA, USA) and Bak (1:2000; Upstate, Charlottesville, VA, USA). Cytosolic and mitochondrial fractions were also processed by Western blot and membranes blotted with Bax, Bim, Cyt *c* (1:2000; BD PharMingen, San Diego, CA, USA) and Smac/Diablo (1:1000; Chemicon, Temecula, CA, USA). Mouse monoclonal antibody to α-Tubulin (1:50000; Sigma, St. Louis, MO, USA) was used as a loading control for total protein extracts and cytosolic fractions. A mouse monoclonal antibody to OxPhos Complex V subunit α (1:5000; anti-ATP synthase subunit α; Molecular Probes, Eugene, OR, USA) was used as a loading control and marker for the mitochondrial fraction. After primary antibody incubation, membranes were washed twice with TBS-T and incubated for 1 h at room temperature with horseradish peroxidase-conjugated antibody (Promega, Madison, WI, USA), and the reaction was finally visualized with the Western Blotting Luminol Reagent (Santa Cruz Biotechnology, CA, USA). Western blot replicates were scanned and quantified using the Phoretix 1D Gel Analysis (Phoretix International Ltd., Newcastle, UK).

Immunoprecipitation

Protein (200 µg) obtained from frozen striata (n = 5 for each condition) was incubated overnight at 4ºC on a rotary mixer with anti-Bcl2 (1:50), anti-Bax (1:100) or anti-Bak (1:100) polyclonal antibodies diluted in lysis buffer and immunoprecipitation was performed as described previously.[10] Blots were immunostained with either an antibody against Bim (1:2000), Bcl-2 (1:500; monoclonal antibody; Transduction Laboratories) or Bcl-x_L (1:500). To ensure that Bcl-2, Bax and Bak were correctly immunoprecipitated, membranes were incubated overnight at 4ºC with anti-Bcl-2 (1:2000), anti-Bax (1:1000) or anti-Bak (1:2000) antibodies.

Statistical analysis

Developmental profiles of Bcl-2 family proteins in the striatum and cortex of wild-type and Bax KO mice were compared using two-way analysis of variance (ANOVA) followed by Scheffe´s post hoc test. Results were normalized to the mean of values obtained in wild-type mice at P3 (100%). Levels of Bcl-2 family proteins in the QUIN-injected striata were compared to those in the striata contralateral (non-injected) to

the lesion using Student's *t* test for independent samples. Data were expressed as a percentage of control (non-injected contralateral striata) values.

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<u>TERCER TRABAJO:</u> *"BH3-only Proteins Bid and BimEL are Differentially Involved in Neuronal Dysfunction in Mouse Models of Huntington's Disease".*

(Aceptado para su publicación en Journal of Neuroscience Research)

OBJETIVO 2.- Estudio de la regulación de las proteínas de la familia de Bcl-2 en los procesos de muerte neuronal en el núcleo estriado.

2.3.- Análisis de la regulación de las proteínas de la familia de Bcl-2 en un modelo transgénico de la enfermedad de Huntington y su posible modulación mediante los niveles endógenos de BDNF.

Dado que observamos que las proteínas de la familia de Bcl-2 regulan la muerte estriatal en un modelo agudo de de la enfermedad de Huntington, nos planteamos estudiar su participación en un modelo crónico de esta enfermedad, el ratón transgénico R6/1, el cual presenta una degeneración estriatal paulatina. Además, dado que el BDNF es el que presenta un mayor efecto neuroprotector en las neuronas estriatales *in vitro* e *in vivo*, el estudio se realizó en paralelo con el modelo desarrollado previamente en nuestro laboratorio, el ratón R6/1:BDNF+/-, lo que nos permitió evaluar la implicación de los niveles de BDNF en esta regulación. Así, se cumplía con los objetivos específicos del punto 2.3. El estudio de los cambios en las proteínas de esta familia constituye una herramienta extremadamente útil para la identificación de nuevas dianas que detengan la muerte neuronal, así como para la evaluación de los efectos terapéuticos de diferentes tratamientos en los modelos de enfermedad de Huntington.

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BH3-Only Proteins Bid and Bim_{EL} Are Differentially Involved in Neuronal Dysfunction in Mouse Models of Huntington's Disease

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Apoptosis, a cell death mechanism regulated by Bcl-2 family members, has been proposed as one of the mechanisms leading to neuronal loss in Huntington's disease (HD). Here we examined the regulation of Bcl-2 family proteins in three different mouse models of HD with exon 1 mutant huntingtin: the R6/1, the R6/1:BDNF+/−, and the Tet/HD94 in which the huntingtin transgene is controlled by the tetracycline-inducible system. Our results disclosed an increase in the levels of the BH3-only proteins Bid and Bim_{EL} in the striatum of HD mouse models that was different depending on the stage of the disease. At 16 weeks of age, Bid was similarly enhanced in the striatum of R6/1 and R6/1:BDNF+/− mice, whereas Bim_{EL} protein levels were enhanced only in R6/1:BDNF+/− mice. In contrast, at later stages of the disease, both genotypes displayed increased levels of Bid and Bim_{EL} proteins. Furthermore, Bax, Bak, Bad, Bcl-2, and Bcl-x_L proteins were not modified in any of the points analyzed. We next explored the potential reversibility of this phenomenon by analyzing conditional Tet/HD94 mice. Constitutive expression of the transgene resulted in increased levels of Bid and Bim_{EL} proteins, and only the Bid protein returned to wild-type levels 5 months after mutant huntingtin shutdown. In conclusion, our results show that enhanced Bid protein levels represent an early mechanism linked to the continuous expression of mutant huntingtin that, together with enhanced Bim_{EL}, may be a reporter of the progress and severity of neuronal dysfunction.

Key words: BDNF; HD94 mice; R6/1 mice; striatum

Huntington's disease (HD) is a neurodegenerative disorder caused by a dominantly heritable expansion of a trinucleotide CAG repeat in the coding region of the HD gene, which results in long stretches of polyglutamine (polyQ) in the N-terminal portion of the protein huntingtin (htt; Huntington's Disease Collaborative Research Group, 1993). Although htt is ubiquitously expressed throughout the central nervous system (Strong et al., 1993; Bhide et al., 1996), its mutation affects most prominently the striatum, with cortical atrophy as the disease progresses (de la Monte et al., 1988; Mann et al., 1993), leading to motor dysfunctions, cognitive impairments, and emotional disturbances (Martin and Gusella, 1986; Bates et al., 2002). To date, the intrinsic neuronal mechanisms that could account for this specific neurodegeneration are not known.

A pathological cross-talk between the nucleus and the mitochondria has been proposed as a mechanism involved in neuronal death and dysfunction in HD (Sawa, 2001). Furthermore, mitochondria have a pivotal role in the regulation of apoptosis that has been suggested as a mechanism of neuronal death in HD (Dragunow et al., 1995; Thomas et al., 1995; Petersen et al., 1999; Portera-Cailliau et al., 1995). Apoptosis involves

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changes in mitochondrial membrane permeabilization leading to the release of proapoptotic molecules such as cytochrome c (Cyt c) or Smac/Diablo, resulting in the activation of caspases, such as caspase-3 (Liu et al., 1996; Zou et al., 1997; Li et al., 1998). Apoptosis-inducing factor (AIF) can also be released, leading to a caspase-independent apoptosis (Susin et al., 1999). Bcl-2 family members are among the proteins that can regulate mitochondrial membrane permeabilization (Polster and Fiskum, 2004; Ward et al., 2004). These proteins are classified into three subfamilies: antiapoptotic members such as Bcl-2 and Bcl-x_L; proapoptotic proteins of the Bax subfamily such as Bax and Bak, which act as apoptosis executers; and the BH3-only family such as Bim, Bid, and Bad, which have been proposed as initiators of apoptosis (Cory and Adams, 2002). Proapoptotic proteins cause mitochondrial dysfunction, leading to the release of apoptogenic factors, whereas antiapoptotic proteins prevent those events. Therefore, the balance between the anti- and proapoptotic mechanisms seems to set tissue homeostasis (Cory and Adams, 2002; Benn and Woolf, 2004).

The development of transgenic mice has allowed the study of how the expression of distinct forms of mutant htt induces HD-like phenotype. R6/1 mice, which express exon 1 with 115 CAG repeats, show decreased striatal volume and develop motor dysfunctions, although no changes in striatal cell number have been observed (Mangiarini et al., 1996). We have recently developed a mouse that expresses the same mutant htt as R6/1 but with low levels of the neurotrophin brain-derived neurotrophic factor (BDNF) (R6/1:BDNF+/−) showing that the reduction in the trophic support advances the onset of motor dysfunctions, with more severe motor abnormalities and loss of striatal neurons (Canals et al., 2004). Similarly, R6/2 mice expressing exon 1 with 150 CAG repeats show reduced levels of endogenous BDNF (Zhang et al., 2003a) and an earlier onset and more severe symptoms than R6/1 mice (Mangiarini et al., 1996). In these animals, a sequential alteration in Bcl-2 family proteins has been suggested to play a role in neuronal cell death (Zhang et al., 2003b). Furthermore, we have shown that BDNF prevents the death of striatal neurons by regulating Bcl-2 family members in an excitotoxic model of HD (Perez-Navarro et al., 2005). Therefore, we asked whether differential changes in protein levels of Bcl-2 family members could account for the more severe symptoms and rapid progression observed in R6/1:BDNF+/− compared with R6/1 mice. Moreover, to study a possible direct regulation of Bcl-2 family members by mutant htt expression, we examined the levels of these proteins in a conditional mouse model of HD (Tet/HD94) in which the expression of mutant htt can be turned off (Yamamoto et al., 2000).

MATERIALS AND METHODS

Mouse HD Models

R6/1 heterozygous transgenic mice expressing exon-1 mutant huntingtin with 115 CAG repeats were obtained from Jackson Laboratory (Bar Harbor, ME). These animals were cross-mated with BDNF heterozygous mice (Ernfors et al., 1994) to obtain R6/1:BDNF+/− mice, as described previously (Canals et al., 2004). Conditional Tet/HD94 mice express a chimeric mouse/human exon 1 with a polyQ expansion of 94 repeats under the control of the bidirectional tetO responsive promoter (Yamamoto et al., 2000). To turn off mutant huntingtin expression, 17-month-old wild-type and Tet/HD94 mice were treated with doxycycline in drinking water during 5 months (2 mg/ml for 4 months followed by 0.5 mg/ml for 1 month; Diaz-Hernandez et al., 2005). Some animals were followed without intervention (gene-ON group). All mice used in the present study were housed together in numerical birth order in groups of mixed genotypes, and data were recorded for analysis by microchip mouse number. Experiments were conducted blindly with respect to genotype. Animals were killed by decapitation under deep CO_2 anesthesia at 8, 12, 16, or 30 weeks (R6/1 and R6/1:BDNF+/− mice) or at 22 months (Tet/HD94 mice) of age.

Mice were genotyped by polymerase chain reaction as described previously (Yamamoto et al., 2000; Canals et al., 2004). The animals were housed with access to food and water ad libitum in a colony room kept at a constant temperature (19–22°C) and humidity (40–50%) on a 12:12-hr light/dark cycle. All animal-related procedures were in accordance with the National Institutes of Health *Guide for the care and use of laboratory animals* and were approved by the local animal care committee of the Universitat de Barcelona (99/01) and the Generalitat de Catalunya (99/1094).

Subcellular Fractionation

Both cytosolic and mitochondrial fractions were extracted from fresh striatal samples as previously described (Zhang et al., 2003b). Tissue was gently homogenized in buffer A (250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 2 mM EDTA, and 20 mM HEPES) plus protease inhibitors [2 mM phenylmethylsulphonyl fluoride (PMSF), 1 mg/μl aprotinin, 1 mg/μl leupeptin, and 1 mM sodium orthovanadate] by passages through a 21G × 1 9/16-in. 0.8- by 40-mm and a 25G × 5/8-in. 0.5- by 16-mm needle fitted on a 2-ml syringe. 1) The resulting homogenates were centrifuged at 500*g* for 5 min at 4°C. 2) Supernatants were collected and centrifuged at 13,000*g* for 20 min at 4°C. 3) The resulting pellets were washed once in buffer A and centrifuged at 13,000*g* for 20 min at 4°C to obtain the mitochondrial fraction. 4) The resulting supernatants from 2 were further centrifuged at 100,000*g* for 60 min at 4°C. The supernatants from 4 were designated *cytosolic fractions*.

Western Blot Analysis

Striatum and cortex were dissected out and homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 10% glycerol, 1% Triton X-100, 150 mM NaCl, 100 mM NaF, 5 μM $ZnCl_2$, and 10 mM EGTA plus protease inhibitors (2 mM PMSF, 1 mg/μl aprotinin, 1 mg/μl leupeptin, and 1 mM sodium orthovanadate) to obtain total protein extracts. To analyze the protein levels of Bcl-2 family members, Western blotting was performed as described elsewhere

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(Perez-Navarro et al., 2005). Total protein extracts and cytosolic or mitochondrial fractions were resolved in denaturing polyacrylamide gel using the Mini-protean system II (Bio-Rad, Hercules, CA). Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) and washed twice in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). After blocking at room temperature for 1 hr, membranes were blotted with the following antibodies: Bax and Bad (Cell Signaling, Beverly, MA), Bcl-x_L and Bcl-2 (Transduction Laboratories, Lexington, KY), Bim (Stressgen, San Diego, CA), Bak (Upstate, Charlottesville, VA), Bid (R&D Systems, Minneapolis, MN) and antibody generously provided by Dr. Krajewski; Krajewska et al., 2002), Cyt c (BD PharMingen, San Diego, CA), Smac/Diablo (Chemicon, Temecula, CA), AIF (Chemicon), fodrin (nonerythroid α-spectrin; Chemicon), LAMP1 (Sigma, St. Louis, MO), or Beclin 1 (Transduction Laboratories). Mouse monoclonal antibody to α-tubulin (Sigma) was used as a loading control for total protein extracts and cytosolic fractions. A mouse monoclonal antibody to OxPhos Complex V subunit α (anti-ATP synthase subunit α; Molecular Probes, Eugene, OR) was used as a loading control and marker for the mitochondrial fraction. After primary antibody incubation, membranes were washed twice with TBS-T and incubated for 1 hr at room temperature with horseradish peroxidase-conjugated antibody (Promega, Madison, WI), and the reaction was finally visualized with the Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA). Western blot replicates were scanned and quantified using Phoretix 1D Gel Analysis (Phoretix International Ltd., Newcastle, United Kingdom).

Caspase-3 Activity Assay

Proteins from fresh striata were prepared by homogenization in lysis buffer as described elsewhere (Perez-Navarro et al., 2005). Proteins (20 μg) were incubated in a 96-well plate with an equal volume of 2× protease assay buffer (20 mM PIPES, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, and 10 mM DTT) plus 50 μM of the fluorogenic substrate Ac-DEVD-acf (BD PharMingen). Plates were incubated at 37°C for 75 min, and the increase in fluorescence was monitored (excitation at 400 nm and emission at 505 nm) using a Spectra Max Gemini XS Fluorometer (Molecular Devices, Union City, CA). The data were presented as the ratio of the fluorescence units in BDNF+/−, R6/1, and R6/1:BDNF+/− to those in wild-type mice ± SEM.

Immunohistochemistry

Immunohistochemical analysis was performed as described elsewhere (Perez-Navarro et al., 2005). Briefly, serial coronal cryostat sections (30 μm) through the whole striatum were collected in phosphate-buffered saline as free-floating sections and incubated with antiactive caspase-3 antibody (Cell Signaling) for 16 hr at 4°C. After washing, sections were incubated with biotinylated secondary antibodies (ABC kit; Pierce, Tattenhall, United Kingdom) and then with avidin-biotin complex. Finally, they were developed with 0.05% diaminobenzidine and 0.02% H_2O_2. As negative controls, some sections were processed as described above in the absence of primary antibody. Stereological quantification of caspase-3-positive cells was carried out by using an optical dissector/Cavalieri combination as described elsewhere (Torres-Peraza et al., 2007). All cell counts were performed blind with respect to genotype (n = 4 per each group). Unbiased stereological counts were obtained from the entire neostriatum in the Computer-Assisted Stereology Toolbox (CAST) software (Olympus, Tokyo, Japan). The dissector counting method was employed to analyze coronal sections spaced by 300 μm. The counting frames were randomly sampled.

Quantitative-Polymerase Chain Reaction Assay

Total RNA was extracted from fresh striata using the Total RNA Isolation Nucleospin RNA II Kit (Macherey-Nagel, Duren, Germany). Total RNA (500 ng) was used to synthesize cDNA with random primers with the StrataScript First Strand cDNA Synthesis System (Stratagene, La Jolla, CA), according to the manufacturer's instructions. The cDNA was then analyzed by quantitative (Q)-polymerase chain reaction (PCR) using the following TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA): 18s (Hs99999901_s1), Bid (Mm00626981_m1), and Bim_{EL} (Bcl2l11; Mm00437795_m1). Q-PCR was performed in reaction buffer containing 12.5 μl Brilliant Q-PCR Master Mix (Stratagene), 1.25 μl TaqMan Gene Expression Assays, and 10–20 ng cDNA. Reactions were as follow: 40 cycles of a two-step PCR; 95°C for 30 sec and 60°C for 1 min, after initial denaturation at 95°C for 10 min. All Q-PCR assays were performed in duplicate and repeated in at least three independent experiments. To provide negative controls and exclude contamination by genomic DNA, the reverse transcriptase was omitted in the cDNA synthesis step, and samples were subjected to the PCR in the same way for each TaqMan Gene Expression Assay. The Q-PCR data were analyzed and quantified by the Comparative Quantitation Analysis program of the MxProTM Q-PCR analysis software version 3.0 (Stratagene) with the 18S gene expression as internal loading control. Results were normalized to cDNAs of wild-type mouse striata and expressed as a percentage of these data.

Striatal Knockin wt and Mutant htt Cell Culture and Transfection

Conditionally immortalized wild-type $STHdh^{Q7/Q7}$ and mutant $STHdh^{Q111/Q111}$ striatal neuronal progenitor cell lines expressing endogenous levels of normal and mutant htt with seven and 111 glutamines, respectively, have been described previously (Trettel et al., 2002). Cells were grown at 33°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 2 mM L-glutamine, and 400 μg/ml G418 (Geneticin; Invitrogen, Carlsbad, CA).

Both wild-type and mutant striatal cell lines were transfected using Lipofectamine 2000 as instructed by the manufacturer. The Mouse Bid subcloned into pCDNA3 expression vector (Invitrogen) was supplied by ADDGENE (Waltham, MA; Addgene plasmid 8774; Zha et al., 2000), and the Human Bim_{EL} subcloned into pCDNA3 expression vector (Invitrogene) was generously provided by Dr. Wang (Yama-

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guchi and Wang, 2002). Bid and Bim_{EL} protein levels were examined by Western blot 24 hr after transfection. Cell lysis and protein extraction were performed as described elsewhere (Gines et al., 2006). Membranes were incubated with anti-Bid, anti-Bim, and antiactin antibodies (as loading control; MP Biomedicals, Inc., Solon, OH) and developed as described above.

Cell Viability

Cell viability was assessed 24 hr after transfection by using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay as described elsewhere (Xifro et al., 2005). Briefly, cells were incubated with MTT (0.2 mg/ml) for 1 hr at 33°C. The blue formazan derivative was solubilized in 500 µl dimethyl sulfoxide, and the dual wavelength was measured at 560 nm and 620 nm in a µQuant Microplate Spectrophotometer (BioTek Instruments, Inc. Winooski, VT). Data were quantified in KCjunior analysis software (BioTek) and presented as percentages of lipofectamine-treated wild-type or mutant cells. All values were obtained from three independent experiments repeated four times.

Statistical Analysis

To analyze changes in tested proteins together between genotypes as well as ages and between genotypes and different brain regions, two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test was performed. The other data were analyzed by one-way ANOVA followed by Bonferroni's post hoc test.

RESULTS

Bid and Bim_{EL} Protein Levels Are Increased in the Striatum of R6/1 and R6/1:BDNF+/− Mice

To identify the role of Bcl-2 family proteins in the neuronal dysfunction observed in HD mouse models, we analyzed their levels by Western blot in the striatum of 16-week-old R6/1 and R6/1:BDNF+/− mice. At this age, the R6/1:BDNF+/− but not the R6/1 mice show significant motor abnormalities (Canals et al., 2004). No differences were observed in Bcl-2 family protein levels between wild-type and BDNF+/− animals (Fig. 1A). The levels of antiapoptotic proteins Bcl-2 and Bcl-x_L as well as proapoptotic proteins Bax, Bak, and Bad were not modified in R6/1 or R6/1:BDNF+/− mice compared with their littermate controls (Fig. 1A). In contrast, BH3-only proteins Bid and Bim_{EL} were enhanced but with differences between genotypes. Bid protein levels were highly increased in both R6/1 and R6/1:BDNF+/− mice (Fig. 1B,D) and were not accompanied by an increase of the truncated form, tBid, which was not detected in any of the genotypes analyzed (Fig. 1B). In contrast, Bim_{EL} protein levels were modified only in R6/1:BDNF+/− mice (Fig. 1C,E). Among the three Bim isoforms described (Bim_{EL}, Bim_L and Bim_S), we detected only Bim_{EL} (Fig. 1C), according to previous data showing that Bim_{EL} is the dominant isoform in the central nervous system (O'Reilly et al., 2000). Furthermore, Bim_{EL} can be phosphorylated by various kinases, resulting in different physiological responses (Ley et al., 2005; Qi et al., 2006). However, we did not detect any modification in the migration pattern of Bim_{EL} that indicates changes in its phosphorylation status (Harada et al., 2004; Qi et al., 2006).

F1

To study the role of Bcl-2 family proteins in neuronal degeneration further, we analyzed protein levels at 30 weeks of age when motor abnormalities are present in both genotypes but significant loss of striatal neurons is detected only in R6/1:BDNF+/− mice (Canals et al., 2004). As observed in 16-week-old animals, striatal levels of prosurvival proteins Bcl-2 and Bcl-x_L and proapoptotic proteins Bax, Bak, and Bad were not modified (Fig. 2A) in any of the HD models analyzed. In contrast, Bid (Fig. 2A,B) and Bim_{EL} (Fig. 2A,C) protein levels were increased significantly in both genotypes.

F2

To analyze whether changes in Bid and Bim_{EL} began earlier during the progression of the disease, protein levels of both proteins were determined in the striatum of 8- and 12-week-old animals. Protein levels of both Bid and Bim_{EL} were not altered in any of the genotypes examined (8 weeks; Bid levels: wild-type: 100 ± 20; R6/1: 113 ± 22; BDNF+/−: 86 ± 9; R6/1:BDNF+/−: 104 ± 20; Bim_{EL} levels: wild-type: 100 ± 3; R6/1: 118 ± 23; BDNF+/−: 93 ± 19; R6/1:BDNF+/−: 103 ± 20; 12 weeks; Bid levels: wild-type: 100 ± 17; R6/1: 98 ± 19; BDNF+/−: 94 ± 6; R6/1:BDNF+/−: 110 ± 17; Bim_{EL} levels: wild-type: 100 ± 3; R6/1: 103 ± 13; BDNF+/−: 76 ± 3; R6/1:BDNF+/−: 118 ± 20).

Bid and Bim_{EL} Protein Levels Are Not Modified in the Cortex of HD Mouse Models

To determine whether increased levels of Bid and Bim_{EL} proteins were specific to the striatum, the main brain area that degenerates in HD, we examined the levels of these proteins in the cortex, a brain area that can be affected in the late stages of the disease. Thus, protein levels were examined in the cortex of wild-type, BDNF+/−, R6/1, and R6/1:BDNF+/− mice at 30 weeks of age. In contrast to that observed in the striatum, no differences in Bid and Bim_{EL} protein levels were observed between R6/1 or R6/1:BDNF+/− and their littermate controls (Fig. 2D–F), indicating that in HD mouse models these proteins are specifically regulated.

Bid, Bim_{EL}, and Bax Protein Levels Are Increased in the Mitochondrial Fraction of R6/1 and R6/1:BDNF+/− Striatum

To study the possible role of changes in the subcellular localization of Bcl-2 family members, the levels of prosurvival proteins Bcl-2 and Bcl-x_L and proapoptotic proteins Bid, Bim_{EL}, and Bax were analyzed in the mitochondrial fraction extracted from the fresh striatum of 30-week-old animals. Although Bax total protein levels were not modified in the striatum of R6/1 or R6/

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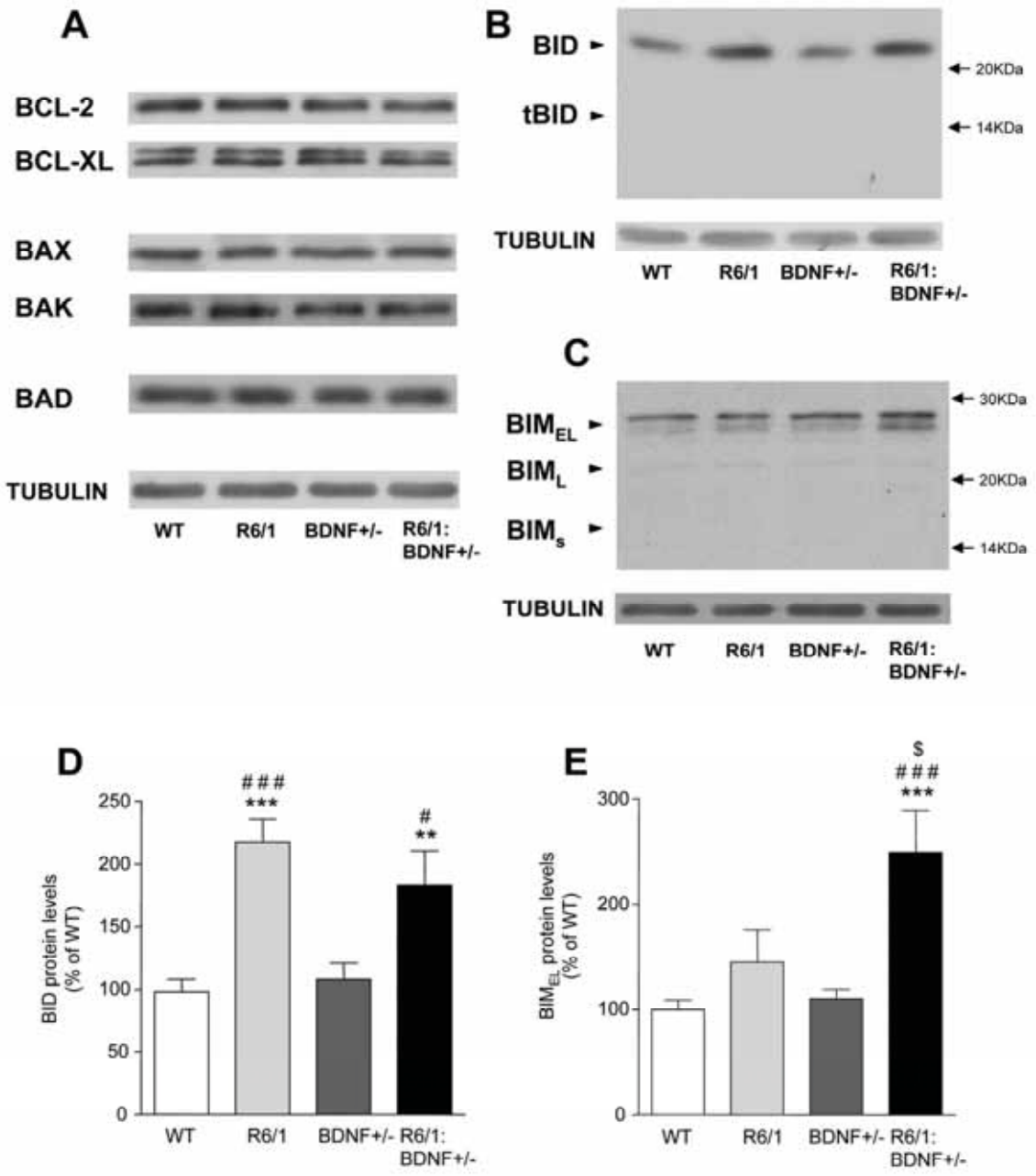

Fig. 1. Bid protein levels are increased in both R6/1 and R6/1:BDNF+/− mice at 16 weeks of age, whereas Bim_{EL} is increased only in the latter. **A–C:** Representative immunoblots showing protein levels of Bcl-2 family members in wild-type (WT), R6/1, BDNF+/−, and R6/1:BDNF+/− mice. **D:** Increase in Bid protein levels in both HD mouse models with respect to their littermate controls. **E:** Specific increase of Bim_{EL} in the R6/1:BDNF+/− mouse at 16 weeks. Values represent the ratio between Bid or Bim_{EL} and α-tubulin levels and are expressed as percentages of WT mice ± SEM for five animals per genotype. Data were analyzed by two-way ANOVA followed by Bonferroni's post hoc test. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with WT mice; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared with BDNF+/− mice; $^{\$}P < 0.01$ compared with R6/1 mice.

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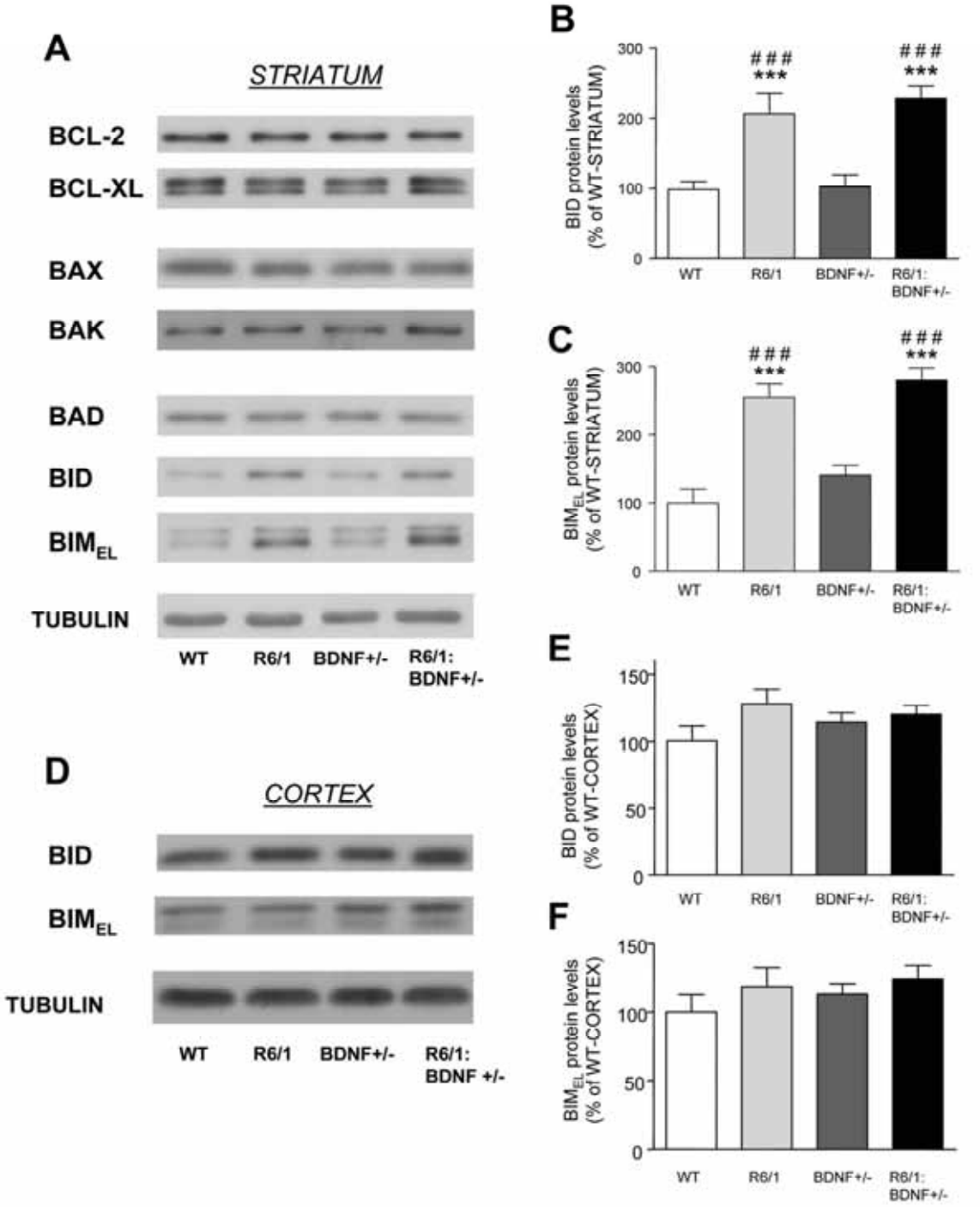

Figure 2.

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1:BDNF+/− mice, mitochondrial Bax levels were significantly increased (Fig. 3A; wild-type: 100 ± 15; R6/1: 147 ± 3★; BDNF+/−: 85 ± 3; R6/1:BDNF+/−: 167 ± 20★; ★$P < 0.05$ compared with wild-type animals). Similarly, Bid (Fig. 3A; wild-type: 100 ± 14; R6/1: 174 ± 30★; BDNF+/−: 117 ± 17; R6/1:BDNF+/−: 187 ± 25★; ★$P < 0.05$ compared with wild-type animals) and Bim_{EL} protein levels (Fig. 3A; wild-type: 102 ± 7; R6/1: 142 ± 11★; BDNF+/−: 92 ± 11; R6/1:BDNF+/−: 144 ± 5★; ★$P < 0.05$ compared with wild-type animals) were increased in the mitochondrial fraction, although high levels of both proteins remained in the cytoplasm (Fig. 3A). In contrast, no changes were detected in the mitochondrial protein levels of the prosurvival members Bcl-2 and Bcl-x_L (Fig. 3A).

F3

No Release of Apoptogenic Factors From Mitochondria Was Detected in the Striatum of HD Mouse Models

We next analyzed whether changes in the localization of the proapoptotic proteins Bid, Bim_{EL}, and Bax were able to induce the release of proapoptotic factors from the mitochondria to the cytosol. To this end, Cyt c, Smac/Diablo, and AIF protein levels were determined by Western blot in both mitochondrial and cytoplasmic fractions obtained from the striatum of 30-week-old mice. No significant differences in the levels of these proteins were detected between HD mouse models and their littermate controls in any of the fractions analyzed (Fig. 3B). Accordingly, caspase-3 activity was not modified in the striatum of R6/1 (104% ± 9% of wild-type levels) and R6/1:BDNF+/− (114% ± 8% of wild-type levels) compared with wild-type or BDNF+/− (96% ± 1% of wild-type levels) striatum. We also examined by Western blot the breakdown products, cleavage of fodrin, a caspase substrate. As expected, a dramatic activation of proteases was not observed, because breakdown products of fodrin were not detected in any of the genotypes analyzed (data not shown). Although changes in the localization of apoptogenic factors and caspase-3 activity were not detected by biochemistry, an immunohistochemical approach allowed the detection of a few positive neurons for active caspase-3 in the striatum of R6/1 (Fig. 4C; 4.6 ± 1 cells/mm^2) and R6/1:BDNF+/− mice (Fig. 4D; 11 ± 2.5 cells/mm^2). In contrast, no labeled neurons for active caspase-3 were observed in the striatum of wild-type (Fig. 4A) and BDNF+/− mice (Fig. 4B). Furthermore, we examined the possible involvement of an autophagic cell death by the analysis of protein levels of LAMP1 and Beclin 1 in the striatum of HD mouse models at 30 weeks of age. No variations were observed in any of the genotypes analyzed (LAMP1: wild-type: 100 ± 21; R6/1: 90 ± 18; BDNF+/−: 97 ± 12; R6/1:BDNF+/−: 103 ± 13; Beclin 1: wild-type:100 ± 12; R6/1:94 ± 1; BDNF+/−: 86 ± 10; R6/1:BDNF+/−: 113 ± 3).

F4

Bid, but Not Bim_{EL}, mRNA Levels Are Up-Regulated in All HD Models

To examine whether increased levels of Bid and Bim_{EL} proteins were directly related to an enhancement in their mRNA expression, Q-PCR was performed in samples obtained from the striatum of 30-week-old animals. Increased levels of Bid mRNA levels were observed in both R6/1 and R6/1:BDNF+/− striatum compared with wild-type and BDNF+/− striatum (Fig. 5A). In contrast, Bim_{EL} mRNA levels were regulated by endogenous BDNF levels. The R6/1 mouse striatum did not show changes compared with wild-type animals, whereas enhanced Bim_{EL} mRNA levels were observed in the striatum of both animals expressing low levels of BDNF, the BDNF heterozygous and the R6/1:BDNF+/− mice (Fig. 5B).

F5

Differential Regulation of Bid and Bim_{EL} Protein Levels in Tet/HD94 Mouse Striatum

To know whether changes in BH3-only proteins depend on the continuous expression of mutant htt, we analyzed these protein levels in the Tet/HD94 mouse, a conditional model of HD (Yamamoto et al., 2000). Striatal Bid and Bim_{EL} protein levels were examined at 22 months in the three different groups, wild-type, Tet/HD94 with no pharmacological intervention (gene-ON) and Tet/HD94 after 5 months of doxycycline administration that turns off the transgene expression (gene-OFF). As observed in R6/1 and R6/1:BDNF+/− striata, both Bid (Fig. 6A) and Bim_{EL} (Fig. 6B) protein levels were increased in the striatum of Tet/HD94 mice with respect to wild-type animals. However, by suppressing transgene expression, only the Bid protein was completely reverted to wild-type levels (Fig. 6).

F6

High Bid or Bim_{EL} Protein Levels Affect the Viability of Striatal Cells Expressing Mutant htt

To ascertain the role of increased levels of Bid and Bim_{EL} on cellular viability, wild-type ($STHdh^{Q7/Q7}$) and

Fig. 2. BH3-only proteins Bid and Bim_{EL} are increased in the striatum, but not in the cortex, of R6/1 and R6/1:BDNF+/− mice at 30 weeks of age. **A:** Immunoblots showing a representative experiment of protein levels of Bcl-2 family members in the striatum of wild-type (WT), R6/1, BDNF+/−, and R6/1:BDNF+/− mice. Increase in Bid (**B**) and Bim_{EL} (**C**) protein levels in the striatum of both mouse models of HD with respect to their littermate controls. Results represent the ratio between Bid or Bim_{EL} and α-tubulin levels and are expressed as percentages of WT ± SEM for five animals per genotype. Data were analyzed by two-way ANOVA followed by Bonferroni's post hoc test. ***$P < 0.001$ compared with WT mice; ###$P < 0.001$ compared with BDNF+/− mice. **D:** Immunoblots, representative of three different experiments, show Bid and Bim_{EL} protein levels in the cortex of wild-type (WT), R6/1, BDNF+/−, and R6/1:BDNF+/− mice are. Bid (**E**) and Bim_{EL} (**F**) protein levels in the cortex of each genotype. Results represent the ratio between Bid or Bim_{EL} and α-tubulin levels and are expressed as percentages of WT ± SEM for five animals per genotype.

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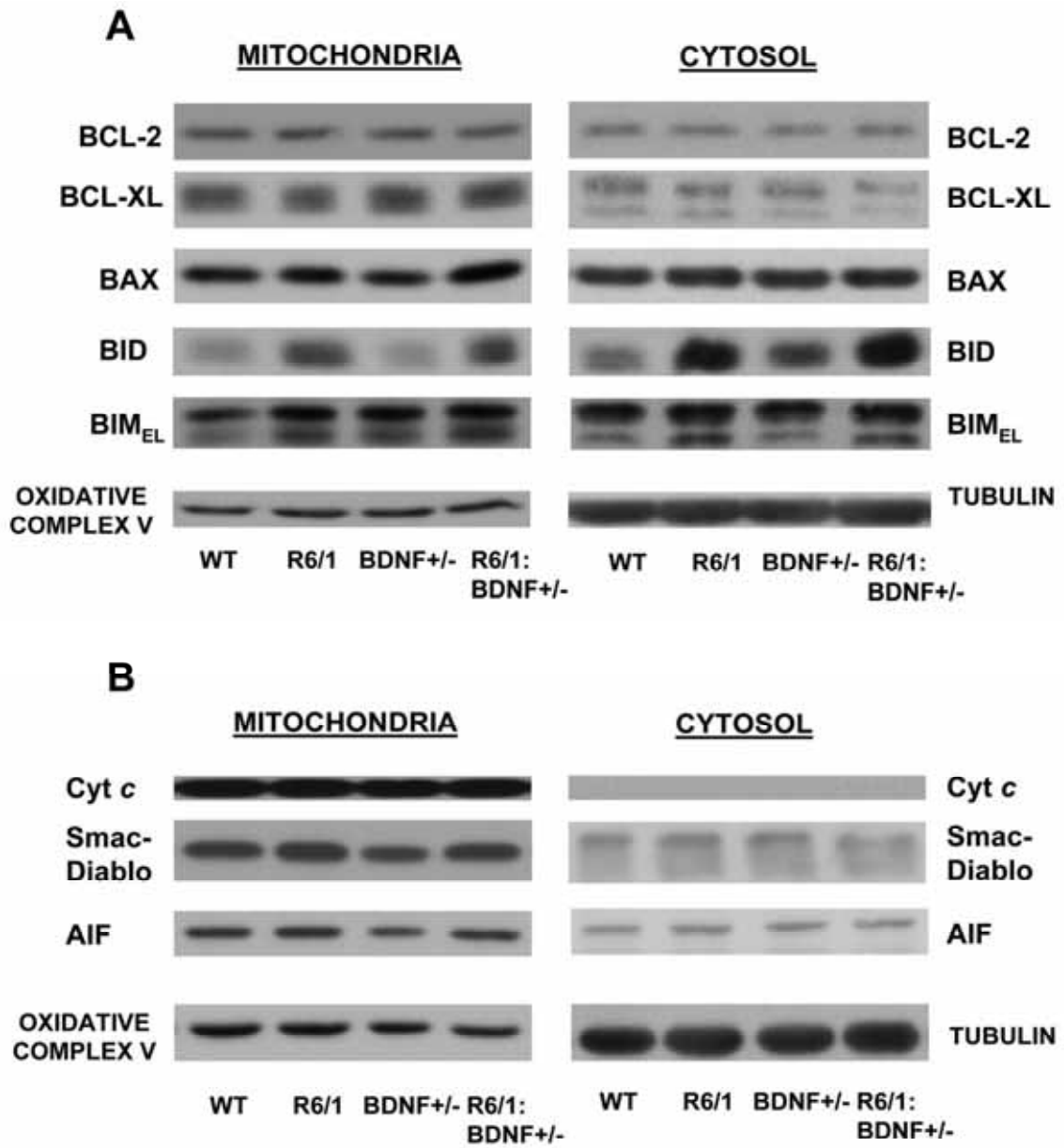

Fig. 3. HD mouse models display an increase in Bax, Bid, and Bim_{EL} protein levels in the mitochondrial fraction that is not accompanied by changes in the localization of mitochondrial apoptogenic factors. **A:** Bcl-2 family proteins were analyzed by Western blot in striatal samples obtained from the striatum of 30-week-old animals (n = 5 for each genotype). Representative immunoblots showing protein levels in mitochondrial and cytosolic fractions of wild-type (WT), R6/1, BDNF+/−, and R6/1:BDNF+/− mice. **B:** Cyt c, Smac/Diablo, and AIF were examined in mitochondrial and cytoplasmic fractions obtained from the striatum of 30-week-old WT, R6/1, BDNF+/−, and R6/1:BDNF+/− mice (n = 5 for each genotype). Immunoblots are representative of three different experiments. The density of each lane was normalized with the corresponding signal of loading controls (oxidative complex V and α-tubulin for mitochondrial and cytoplasmic fractions, respectively).

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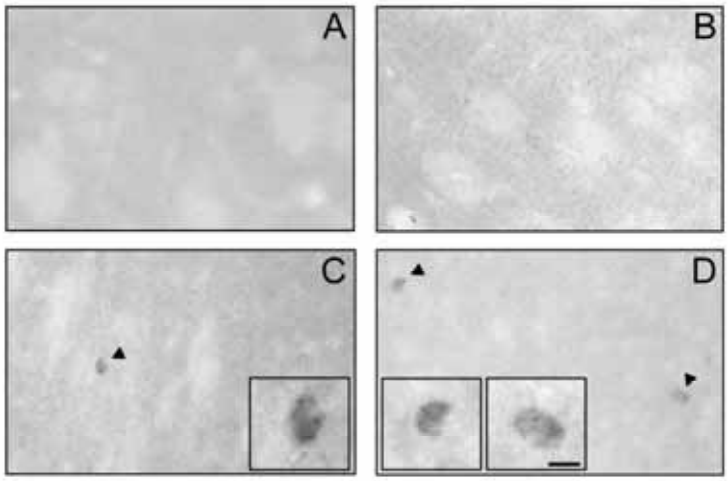

Fig. 4. R6/1 and R6/1:BDNF+/− mice show active caspase-3-positive cells in the striatum. Immunohistochemistry was performed in wild-type (WT), R6/1, BDNF+/−, and R6/1:BDNF+/− mice (n = 3 for each genotype) at 30 weeks of age. Photomicrographs showing the striatum of wild-type (**A**), BDNF+/− (**B**), R6/1 (**C**), and R6/1:BDNF+/− (**D**) mice. **Insets** show high-power images of cells labeled with the antiactive caspase-3 antibody in the striatum of mouse HD models. Scale bar = 30 μm for A–D; 10 μm for insets.

mutant ($STHdh^{Q111/Q111}$) striatal cells were transfected with plasmids expressing Bid or Bim_{EL} proteins. As shown in Figure 7A, Bid and Bim_{EL} protein levels were increased to a similar extent in wild-type and mutant striatal cells 24 hr after transfection. Bid overexpression reduced the viability of both wild-type (by 31% ± 2%) and mutant cells (by 51% ± 5.5%) 24 hr after transfection, with a higher effect on mutant cells (Fig. 7B). In contrast, overexpression of Bim_{EL} protein did not exert any effect on the viability of wild-type cells, whereas mutant striatal cells were moderately affected (by 14% ± 0.3%; Fig. 7B).

DISCUSSION

In the present work, we examined the possible contribution of Bcl-2 family proteins to striatal neuron degeneration in three different mouse models of HD. Our results show that increased levels of Bid and Bim_{EL} together with no modifications in other Bcl-2 family proteins, is a common feature in the striatum of the mouse models analyzed. However, Bid and Bim_{EL} proteins showed a different regulation, insofar as increased Bid protein levels were observed in the early stages of the disease, whereas changes in Bim_{EL} protein appeared later. Nevertheless, the analysis of these proteins in the conditional Tet/HD94 mice disclosed that only changes in Bid protein levels returned to wild-type levels by shutting down mutant htt expression in aged animals. Furthermore, we show that overexpression of full-length Bid or Bim_{EL} was enough to compromise the viability of striatal cells expressing mutant htt.

Here we show that levels of the BH-3-only proteins, Bid and Bim_{EL}, were similarly increased in the striatum of R6/1 and R6/1:BDNF+/− mice in the late stages of the disease (30 weeks), although loss of striatal neurons is observed only in the latter (Canals et al., 2004). In contrast, protein levels of other members of the Bcl-2 family, such as Bax, Bak, Bcl-2, Bcl-x_L, and Bad, were not modified. The specific changes in Bid and Bim_{EL} proapoptotic proteins could be related to their proposed role as cell stress sensors and to the idea that BH-3-nly proteins signal cellular damage (Huang and Strasser, 2000; Fletcher and Huang, 2006). Interestingly, we have also observed that, at earlier stages of the disease (16 weeks), Bid protein levels were similarly enhanced in both genotypes, whereas Bim_{EL} protein levels were increased only in the striatum of R6/1:BDNF+/− mice correlating with the presence of motor dysfunction (Canals et al., 2004). In agreement with our results, it has been previously shown that, in R6/2 mice, changes in the Bid protein occur earlier than the increases of the Bim protein that are observed at a late stage of the disease progression (Zhang et al., 2003b). A deficit in the neurotrophic factor BDNF has been proposed as one of the mechanisms that can participate in the striatal neuron dysfunction induced by mutant htt. BDNF expression (Zuccato et al., 2001, 2003) and its vesicular transport along the microtubules (Gauthier et al., 2004) is reduced in the presence of mutant htt. Furthermore, decreased levels of endogenous BDNF advance the onset of the disease and produce more severe motor impairments (Canals et al., 2004). Here, we present evidence that reduced levels of endogenous BDNF favor the increase of Bim_{EL} protein levels in the striatum at early stages of the disease progression (16 weeks). Therefore, our results point to the idea that increased levels of Bim_{EL} in the R6/1:BDNF+/− striatum may contribute to the acceleration of neuronal dysfunction and degeneration. In support of this suggestion, we show that striatal progenitors expressing full-length htt with 111 CAG repeats are more sensitive than wild-type cells to the presence of high levels of Bim_{EL} protein.

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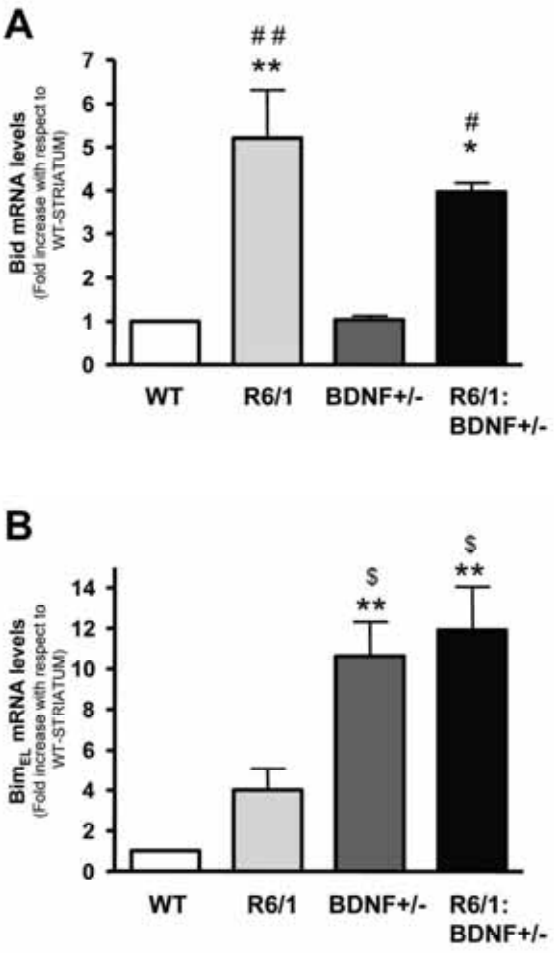

Fig. 5. Enhanced levels of Bid, but not Bim_{EL}, mRNA in the striatum of both R6/1 and R6/1:BDNF+/− mice. Bid and Bim_{EL} mRNA levels were analyzed by Q-PCR in the striatum of 30-week-old animals (n = 5 for each genotype). Bid (**A**) and Bim_{EL} (**B**) mRNA levels in the striatum of wild-type (WT), R6/1, BDNF+/−, and R6/1:BDNF+/− mice. Results were normalized the 18S gene expression and expressed as a percentage of WT ± SEM. Data were analyzed by one-way ANOVA followed by Bonferroni's post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with WT mice; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with BDNF+/− mice; $^{\$}P < 0.05$ compared with R6/1 mice.

The striatum is the main area that degenerates in HD, but cortical atrophy has also been reported in human patients as the disease progresses (Vonsattel and DiFiglia, 1998; MacDonald and Halliday, 2002). Similarly, R6/1 and R6/1:BDNF+/− mice display reduced cortical volume at 30 weeks of age compared with their wild-type littermates (Canals et al., 2004). However, we did not detect changes in Bid and Bim_{EL} protein levels in this brain region, suggesting that these proteins play a specific role in the susceptibility of striatal neurons to mutant htt-induced degeneration. In accordance with our results, proapoptotic members of the Bcl-2 family are differentially regulated in striatal and cortical neurons in response to 3-nitropropionic acid-induced neurodegeneration, a model of HD (Galas et al., 2004).

In the present study we also show a high increase in the levels of full-length Bid protein without the appearance of truncated Bid (tBid). In agreement, other studies show that, in response to several cell death signals, full-length Bid can be localized in mitochondria contributing to the induction of apoptosis (Sarig et al., 2003; Ward et al., 2006). In fact, the present results show that overexpression of full-length Bid protein reduced the viability of wild-type and more severely of mutant striatal cells. Insofar as we observed an increase in full-length Bid protein levels in the mitochondrial fraction obtained from R6/1 and R6/1:BDNF+/− mice striatum, our results suggest that sustained high levels of the full-length Bid during disease progression may contribute to the mitochondrial damage (Beal, 2000; Rego and Oliveira, 2003) and neuronal dysfunction that precede cell death. A previous study performed with total brain extracts from R6/2 mice showed decreased levels of full-length Bid, accompanied by an increase in the tBid protein (Zhang et al., 2003b). In R6/2 mice the disease progresses more rapidly and with more severity than in R6/1 mice (Mangiarini et al., 1996), so the presence of tBid seems to be related to a greater neuronal dysfunction. In addition to increased Bid levels in the mitochondrial fraction, we also observed a slight relocalization of Bim_{EL} and Bax proapoptotic proteins to the mitochondria. However, these changes were not followed by a general apoptotic response downstream of mitochondria in the HD mouse striatum, in that no changes in the localization of Cyt c, Smac/Diablo, or AIF were detected. HD is a slowly progressive disorder, so only a few cells would be expected to undergo cell death in a given period of time, making it difficult to detect these changes. In agreement, we observed only a few neurons showing active caspase-3 labeling in the striatum of R6/1 and R6/1:BDNF+/− mice. Similarly, weak or no immunoreactivity for active caspase-3 has been reported in human brain samples from different HD grades (Vis et al., 2005). Moreover, autophagy, a caspase-independent mechanism of cell death, contributes to striatal degeneration (Kegel et al., 2000). However, we did not detect changes in the autophagic markers LAMP1 (Eskelinen et al., 2003) and Beclin 1 (Gozuacik and Kimchi, 2004) in the striatum of the HD models analyzed.

To study whether increased levels of Bid and Bim_{EL} proteins in the striatum were directly related to mutant htt expression, these proteins were analyzed in a conditional mouse model of HD, the Tet/HD94 mouse (Yamamoto et al., 2000). At 17 months of age Tet/HD94 mice show deficits in motor coordination, a decrease in striatal volume, and a significant decrease

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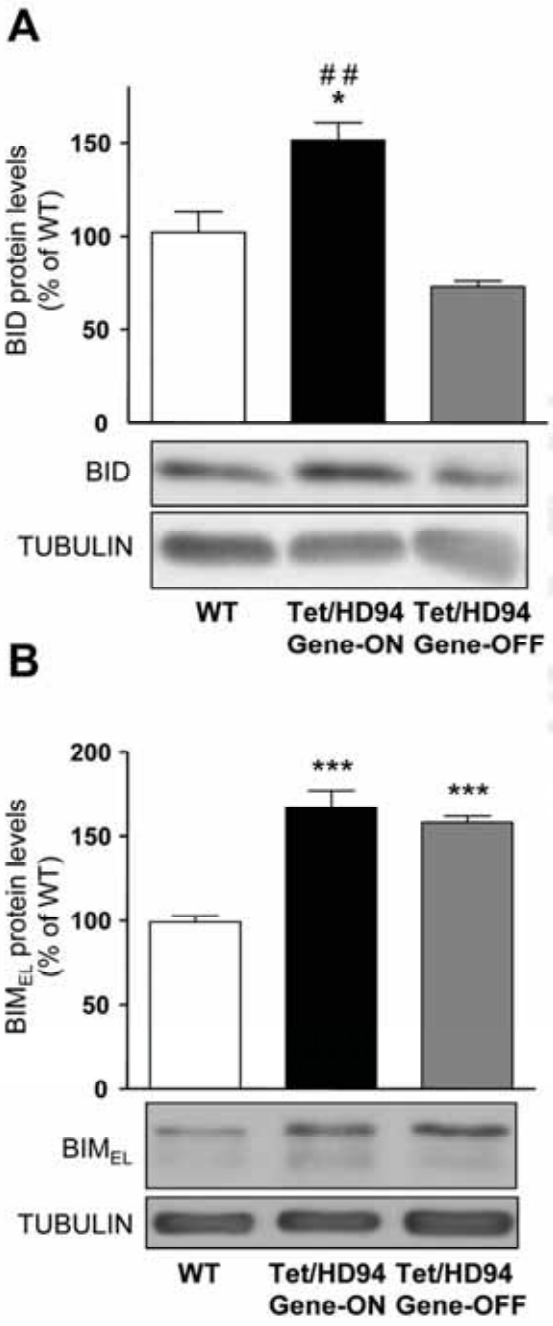
in the number of striatal neurons that progress with age (Diaz-Hernandez et al., 2005). Here we show that, as observed in R6/1 and R6/1:BDNF+/− mice, Tet/HD94 mice displayed increased levels of Bid and



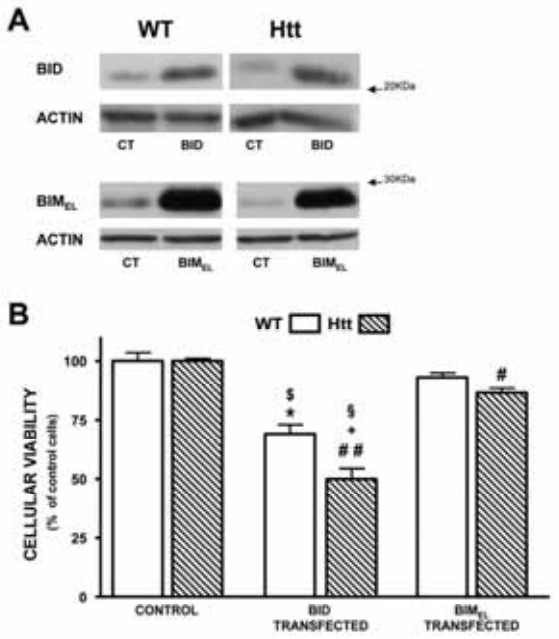


Fig. 7. High expression of Bid, but not Bim_{EL}, reduces the viability of both wild-type and mutant cells. **A:** Immunoblots showing Bid and Bim_{EL} protein levels in wild-type (WT) and mutant cells (Htt) 24 hr after treatment with lipofectamine (CT; control) or 24 hr after transfection with either pcDNA3-Bid (BID) or pcDNA-Bim_{EL} (Bim_{EL}). Membranes were reprobed with antiactin as loading control. **B:** Cell viability assessed by the MTT assay 24 hr after transfection of wild-type (WT) and mutant (Htt) cells with pcDNA3-Bid (BID) or pcDNA-Bim_{EL} (Bim_{EL}). Results are expressed as percentages of lipofectamine-treated WT or mutant cells ± SEM for three independent experiments. Statistical analysis was performed by two-way ANOVA, followed by Bonferroni's post hoc test. $^{*}P < 0.001$ compared with WT treated with lipofectamine; $^{\$}P < 0.001$ compared with WT transfected with Bim_{EL}; $^{\#}P < 0.01$, $^{\#\#}P < 0.001$ compared with Htt treated with lipofectamine; $^{*}P < 0.01$ compared with Htt-transfected with Bim_{EL}; $^{\$}P < 0.01$ compared with WT transfected with Bid.

Bim_{EL} proteins in the striatum at 22 months of age, when striatal neuron loss is about 44% with respect to their control littermates (Diaz-Hernandez et al., 2005). Interestingly, we observed that transgene shutdown

Fig. 6. Differential regulation of Bid and Bim_{EL} protein levels in the striatum of Tet/HD94 mice. Bid and Bim_{EL} protein levels were examined in the striatum of 22-month-old wild-type (WT) and Tet/HD94 mice either with no pharmacological intervention (gene-ON) or after 5 months of transgene shutdown by doxycycline administration (gene-OFF). A and B show the densitometric measures of Bid (**A**) and Bim_{EL} (**B**) normalized to α-tubulin protein levels and expressed as percentages of WT ± SEM for four animals per condition. Data were analyzed by one-way ANOVA followed by Bonferroni's post hoc test. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with WT mice; $^{\#\#}P < 0.01$ compared with Tet/HD94 (gene-OFF) mice. Immunoblots show representative experiments.

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during 5 months completely reverted Bid, but not Bim_{EL}, up-regulation. Under the same conditions, Tet/HD94 mice show a reduction in cell loss and full recovery from their motor deficit (Diaz-Hernandez et al., 2005). Taken together, our results suggest that the reduction in cell loss after turning off the transgene expression could be related, at least in part, to the reduction of Bid protein levels and that increased levels of both Bid and Bim_{EL} proteins are necessary to induce neuronal dysfunction. Furthermore, our results also suggest that two different mechanisms regulate Bid and Bim_{EL} proteins in the presence of mutant htt expression. One of the mechanisms by which mutant htt has been suggested to induce neurodegeneration is by impairing gene transcription (Li and Li, 2004). In this context, mutant htt has been shown to bind to p53. up-regulating its levels in the nucleus as well as its transcriptional activity (Bae et al., 2005). p53 Has been shown to up-regulate the transcription of several proteins related to apoptosis, such as Bid (Sax and El Deiry, 2003). Our results showing that Bid mRNA levels are similarly increased in the R6/1 and R6/1:BDNF+/− mouse striatum suggest that mutant htt may regulate Bid at the transcriptional level, thus explaining why, in the absence of mutant htt expression, Bid protein returns to wild-type levels. However, we cannot rule out that other BH3-only proteins regulated by p53, not examined in the present work, such as Puma and Noxa (Sax and El Deiry, 2003), could also contribute to striatal neuron degeneration. In contrast, the accumulation of high amounts of Bim_{EL} protein seems to be regulated by a slower mechanism. In fact, Bim_{EL} protein can be regulated by different post-translational mechanisms in addition to the regulation at the transcriptional level (Huang and Strasser, 2000). Our results showing that Bim_{EL} protein, but not mRNA, levels are enhanced in the striatum of R6/1 mice suggest that the presence of mutant htt could interfere with pathways involved in Bim_{EL} degradation. Furthermore, we observed enhanced levels of Bim_{EL} mRNA only in mice with low levels of endogenous BDNF (BDNF+/− and R6/1:BDNF+/−) in support to data showing that BDNF down-regulates Bim_{EL} mRNA levels (Li et al., 2006). Reinforcing our hypothesis, only the R6/1:BDNF+/− mice showed enhanced levels of Bim_{EL} protein.

In summary, the data presented here suggest an important role of the BH3-only proteins, Bid and Bim_{EL}, as sensors of striatal neuron stress resulting from the mutant htt expression. In the presence of mutant htt expression, these two proteins seem to be controlled by different mechanisms but possibly with the same consequence, the sensitization of striatal neurons to make them reactive to apoptotic stimulus. In addition, Bim_{EL} and Bid proteins can also act directly by disrupting mitochondrial integrity contributing to the mitochondrial defects described in HD pathogenesis (Beal, 2000; Rego and Oliveira, 2003). Furthermore, these proapoptotic changes can be partially reversed by the suppression of mutant htt expression. Therefore, our results suggest that the study of specific changes in Bcl-2 family protein levels could be a reporter of the progress and severity of neuronal dysfunction and can help to identify new targets to stop neuronal death and to evaluate therapeutic effects of drugs in HD models.

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CUARTO TRABAJO: *"Prosurvival Phospho-AKT/PKB Levels are Increased during the Neurodegenerative Process in the Striatum of Mouse Expressing the N-terminal Exon-1 of Mutant Huntingtin".*

(En preparación)

OBJETIVO 3.- Estudio de los mecanismos de supervivencia activados en respuesta a la toxicidad inducida por la expresión de la huntingtina mutada en modelos transgénicos de la enfermedad de Huntington.

3.1.- Estudio del posible papel neuroprotector de la quinasa AKT frente a los mecanismos apoptóticos inducidos por la expresión de huntingtina mutada.

Como objetivo 3 estudiamos la activación de AKT en los ratones R6/1 y R6/1:BDNF+/-. Comenzamos este trabajo de acuerdo con la hipótesis de que algún tipo de mecanismo anti-apoptótico pudiera estar activado en respuesta a la toxicidad inducida por la htt mutada, bloqueando, o al menos retrasando, las consecuencias de los cambios apoptóticos observados en el trabajo anterior. En el presente trabajo también analizamos si la reducción del factor trófico BDNF era capaz de modular la activación de AKT en el núcleo estriado, y si esta activación permitiría recuperar la viabilidad neuronal tras el silenciamiento de la huntingtina mutada. Nuestro objetivo era evaluar la relevancia de esta vía anti-apoptótica en el contexto de la enfermedad de Huntington, así como analizar su papel como medio efectivo de retrasar la muerte de las neuronas estriatales.

Prosurvival phospho-AKT/PKB levels are increased during the neurodegenerative process in the striatum of mouse expressing the N-terminal exon 1 of mutant huntingtin

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Running title: Increased phospho AKT/PKB levels in HD models.

Keywords: Huntington's disease, neuronal death, BDNF, PI3-K, GSK3β, FoxO.

Abbreviations: BDNF, Brain derived neurotrophic factor; FoxO, Forkhead factors; GSK3, glycogen synthase kinase 3; htt, huntingtin; HD, Huntington's disease; mhtt, mutant huntingtin; polyQ, polyglutamine.

Abstract

In mouse models expressing exon 1 of mutant huntingtin (mhtt) the reduction in the striatal volume is the result of a massive decreased neuropil because neuronal death is minimal. Survival mechanisms to prevent cell death may be therefore activated. In this study we analyzed the levels of the phospho-Akt protein, which is involved in promoting cell survival, in four different exon 1 mutant huntingtin mouse models: the R6/1, the R6/1:BDNF +/-, the R6/2 and the Tet/HD94 in which the mutant transgene is controlled by the tetracycline inducible system. Our results show that in the R6/1 and R6/1: BDNF +/- mice, phospho-Akt levels were specifically increased in the striatum during different stages of the disease progression. These enhanced levels of phospho-Akt were still detected at the latest stages of the neurodegenerative process (30 weeks) and were related to the inactivation of the pro-apoptotic proteins GSK3β and FoxO1. In addition, the R6/2 and the Tet/HD94 mouse models also displayed enhanced levels of phospho-Akt in the striatum at late stages of the disease progression. Interestingly, in the Tet/HD94 mouse, phospho-Akt levels almost returned to wild-type levels after mhtt shut-down. In conclusion, our results show that activation of the Akt/PKB pathway is a common mechanism taking place in neurons expressing exon 1 mhtt. Furthermore, we suggest that high levels of phospho-Akt may prevent cell death and allow the recovery of neuronal viability after mhtt silencing.

Introduction

Huntington's disease (HD) is a neurodegenerative disorder characterized by motor dysfunction, cognitive impairments and emotional disturbances (Martin and Gusella, 1986). The causative mutation of HD is a dominantly heritable expansion of a trinucleotide CAG repeat in the coding region of the HD gene, which results in long stretches of polyglutamine (polyQ) in the N-terminal portion of the huntingtin protein (htt; HDCRG, 1993). Htt is ubiquitously expressed throughout the central nervous

system (Strong et al., 1993; Bhide et al., 1996), but its mutation involves a selective death of medium spiny neurons of the striatum and cortical atrophy at later stages of disease progression (DeLa Monte et al., 1988; Mann et al., 1993). The intrinsic neuronal mechanisms that account for this specific neurodegeneration are still not known, and different mechanisms such as excitotoxicity, mitochondrial dysfunction and lack of trophic support have been related to the development of the neurodegenerative process (reviewed in: Perez-Navarro et al., 2006).

One pathological hallmark of HD is the presence of htt inclusion bodies in neurons. However, the exact role of these aggregates in HD pathogenesis is still under discussion (Davies y col., 1997; DiFiglia y col., 1997; Kim y col., 1999; Leavitt y col., 1999; Saudou y col., 1998). It has been shown that aggregation, for any given polyQ, is greater for the N-terminal truncated protein than for the full-length protein (Cooper et al., 1998; Hackam et al., 1998; Martindale et al., 1998). Interestingly, htt can be cleaved by different proteases (Wellington et al., 2000a; Kim et al., 2001; Lunkes et al., 2002; Gafni et al., 2004) and several lines of evidence indicate that htt cleavage and nuclear localization are necessary to induce degeneration (Sadou et al., 1998; Kim et al., 1999; Peters et al., 1999; Wellington et al., 2000b). In addition, htt phosphorylation can also participate in the pathological process. In particular, phosphorylation of mhtt at serine 421 (S421) and serine 434 (S434) blocks its toxic effects on striatal neurons (Humbert et al., 2002; Luo et al., 2005).

The AKT has been proposed as an important neuroprotective pathway in HD. This protein is one of the serine/threonine kinase that phosphorylates the S421 of mutant htt inhibiting its toxicity (Humbert et al., 2002). AKT is ubiquitously expressed in mammals, and although it is initially present at low levels in the adult brain (Owada et al., 1997) its expression increases dramatically in neurons during cellular stress or

injury (Owada et al., 1997; Kang et al., 2004; Chong et al., 2005). In accordance with this, enhanced AKT signaling appears as an early prosurvival response in the striatum of the knock-in mouse model *Hdh*$^{Q111/Q111}$ and in the striatal cell line ST*Hdh*$^{Q111/Q111}$ model of HD (Gines et al., 2003). However, in an acute HD rat model, with the intrastriatal injection of lentiviral vectors expressing N-terminal fragments of htt with a pathological stretch of 82Q, both AKT and phospho-AKT levels are down-regulated during neuronal dysfunction (Colin et al., 2005). Moreover, AKT has been found cleaved and presumably permanently deactivated by caspase-3 in HD postmortem human brains (Colin et al., 2005).

Interestingly, a recent report demonstrates that AKT, apart from phosphorylation of the S421 of mutant htt, elicits neuroprotection by a different mechanism through arfaptin phosphorylation (Rangone et al., 2005). In addition, once activated, AKT provides cells with multiple survival signals that can also act increasing neuronal survival, for example targeting the proapoptotic Bcl-2 related protein, BAD (Del Peso et al., 1997; Datta et al., 1997), affecting Forkhead (FoxO) factors (Brunet et al., 1999; Kops and Burgering 1999; Biggs et al., 1999; Rena et al., 1999) and p53 family (Mayo et al., 2001; Gottlieb et al., 2002), and acting through glycogen synthase kinase 3 inactivation (GSK3; Pap and Cooper., 1998).

In this work we analyzed AKT expression and activity in four different models which specifically express the exon 1 of mhtt with different CAG repeats: The well-studied model of HD, R6/1 mice, expressing exon 1 with 115 CAG repeats; the R6/1:BDNF+/- mouse, that expresses the same mhtt as R6/1 but with low levels of the neurotrophin BDNF (Canals et al., 2004); the R6/2 mouse, expressing exon 1 with 150 CAG repeats and reduced levels of endogenous BDNF (Zhang et al., 2003a); and a conditional mouse model of HD, Tet/HD94, in which the expression of mhtt can be

turned off (Yamamoto et al., 2000). Both R6/2 and R6/1:BDNF+/- mice show more severe symptoms than R6/1 and Tet/HD94 mice (Mangiarini et al., 1996; Canals et al., 2004; Diaz-Hernandez et al., 2005). However, all the models used in the present work display increased levels of the pro-apoptotic proteins of Bcl-2 family members (Zhang et al., 2003b; Garcia-Martinez et al., 2007), although weak or no apoptotic cell death have been observed (Mangiarini et al., 1996; Canals et al., 2004; Diaz-Hernandez et al., 2005; Garcia-Martinez et al., 2007). Therefore, we asked whether the reduction in trophic support could intensify the activation of the AKT pathway in response to mhtt, and if this activation could account for avoiding massive cell death within the striatum allowing the recovery of neuronal viability and function after mhtt turn off.

Materials and methods

Mouse HD models

R6/1 and R6/2 heterozygous transgenic mice expressing exon-1 mutant huntingtin with 115 and 150 CAG repeats, respectively, were obtained from Jackson Laboratory (Bar Harbor, ME, USA). R6/1 mice were cross-mated with BDNF heterozygous mice (Ernfors et al., 1994) to obtain R6/1:BDNF+/- mice, as previously described (Canals et al., 2004). Conditional Tet/HD94 mice express a chimeric mouse/human exon 1 with a polyQ expansion of 94 repeats under the control of the bidirectional tetO responsive promoter (Yamamoto et al., 2000). To turn off mutant huntingtin expression, 17 month-old wild-type and Tet/HD94 mice were treated with doxycycline in drinking water during 5 months (2 mg/ml for 4 months followed by 0.5 mg/ml for 1 month; Diaz-Hernandez et al., 2005). Some animals were allowed without intervention (gene-on group). All mice used in the present study were housed together in numerical birth order

in groups of mixed genotypes and data were recorded for analysis by microchip mouse number. Experiments were conducted in a blind-coded respect to genotype. Mice were genotyped by polymerase chain reaction as described previously (Yamamoto et al., 2000; Canals et al., 2004). The animals were housed with access to food and water *ad libitum* in a colony room kept at a constant temperature (19-22°C) and humidity (40-50%) on a 12:12 h light/dark cycle. All animal-related procedures were in accordance with the National Institute of Health Guide for the care and use of laboratory animals and approved by the local animal care committee of the Universitat de Barcelona (99/01) and the Generalitat de Catalunya (99/1094).

Total protein extraction

Animals were deeply anesthetized and killed by decapitation at 12, 16 or 30 weeks (R6/1 and R6/1:BDNF+/- mice), 12 weeks (R6/2 mice) or at 22 months (Tet/HD94 mice) of age. Striatum, cortex and hippocampus were quickly removed and homogenized in lysis buffer [50mM Tris–HCl (pH 7.5), 10% glycerol, 1% Triton X-100, 150 mM NaCl, 100 mM NaF, 5µM $ZnCl_2$ and 10mM EGTA] plus protease inhibitors [phenylmethylsulphonyl fluoride (2mM), aprotinin (1mg/µl), leupeptin (1mg/µl) and sodium orthovanadate (1mM)].

Western blot analysis

To examine striatal, cortical and hippocampal protein levels, Western blot was performed as described elsewhere (Perez-Navarro et al., 2005). Cell lysates were resolved in denaturing polyacrylamide gel using the Mini-protean system II (BioRad). Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore) and washed twice in Tris-Buffered saline containing 0.1% Tween-20 (TBS-

T). After blocking at room temperature for 1h, membranes were blotted with the primary antibodies anti-: Phospho-AKT (Ser473), phospho-FoxO (Ser256), phospho-GSK3β (Ser9), FoxO1 and GSK3β (Cell Signaling, Beverly, MA, USA); PKBα/AKT, (BD Transduction Laboratories, Lexington, KY, USA). Mouse monoclonal antibody to α-Tubulin, (Sigma, St. Louis, MO, USA), was used as a loading control. After primary antibody incubation, membranes were washed twice with TBS-T and incubated for 1 h at room temperature (r.t.) with horseradish peroxidase-conjugated antibody (1:2000; Promega), and the reaction was finally visualized with the Western Blotting Luminol Reagent (Santa Cruz Biotechnology, California, USA). Western blot replicates were scanned and quantified using the Phoretix 1D Gel Analysis (Phoretix International Ltd., Newcastle, UK).

Statistical analysis

To analyze changes of tested proteins together between genotypes as well as ages, two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test was performed. The other data were analyzed using one-way ANOVA followed by Bonferroni's post-hoc test.

Results

The striatum of R6/1 and R6/1:BDNF+/- mice exhibit enhanced AKT activation during the neuropathological process

To analyze the *in vivo* regulation of AKT in the pathological progression observed in two exon 1 mhtt mouse models with different BDNF endogenous levels, we analyzed AKT and phospho(S473)-AKT levels by western blot in the striatum of R6/1 and R6/1:BDNF+/- mice at 12, 16 and 30 weeks.

At 12 weeks, the onset of motor abnormalities in the R6/1:BDNF+/- mouse (Canals et al., 2004), we detected an increase of phospho(S473)-AKT levels in both R6/1 and R6/1:BDNF+/- mice while no changes in the total levels of AKT were detected in any of the genotypes analyzed (Fig. 1A), pointing to an early response of the AKT kinase in response to mhtt expression. Similarly, at 16 weeks, when only the R6/1:BDNF+/- mouse displays motor abnormalities (Canals et al., 2004), we observed an increase of phospho(S473)-AKT levels in both R6/1 and R6/1:BDNF+/- mice (Fig. 1B). This increase was accompanied by a significant enhancement of the phospho(S473)-AKT/AKT ratio (Fig. 1D), since no changes in the total levels of AKT were detected in any of the genotypes analyzed (Fig. 1B).

To further study the role of the AKT pathway during neuronal degeneration, we analyzed phosphorylated and total protein levels at 30 weeks when both genotypes exhibit severe motor abnormalities and a significant loss of striatal neurons is detected only in R6/1:BDNF+/- mice (Canals et al., 2004). Interestingly, the significant increase of phospho(S473)-AKT levels observed in younger animals was still detectable at late stages of the disease (Fig. 1C). Moreover, although a slight rise was detected in the total AKT levels in both HD mice (Fig. 1C), the large increase of phospho(S473)-AKT levels

was also accompanied by a higher enhancement of the phospho(S473)-AKT/AKT ratio than that observed at 12 and 16 weeks (Fig. 1D).

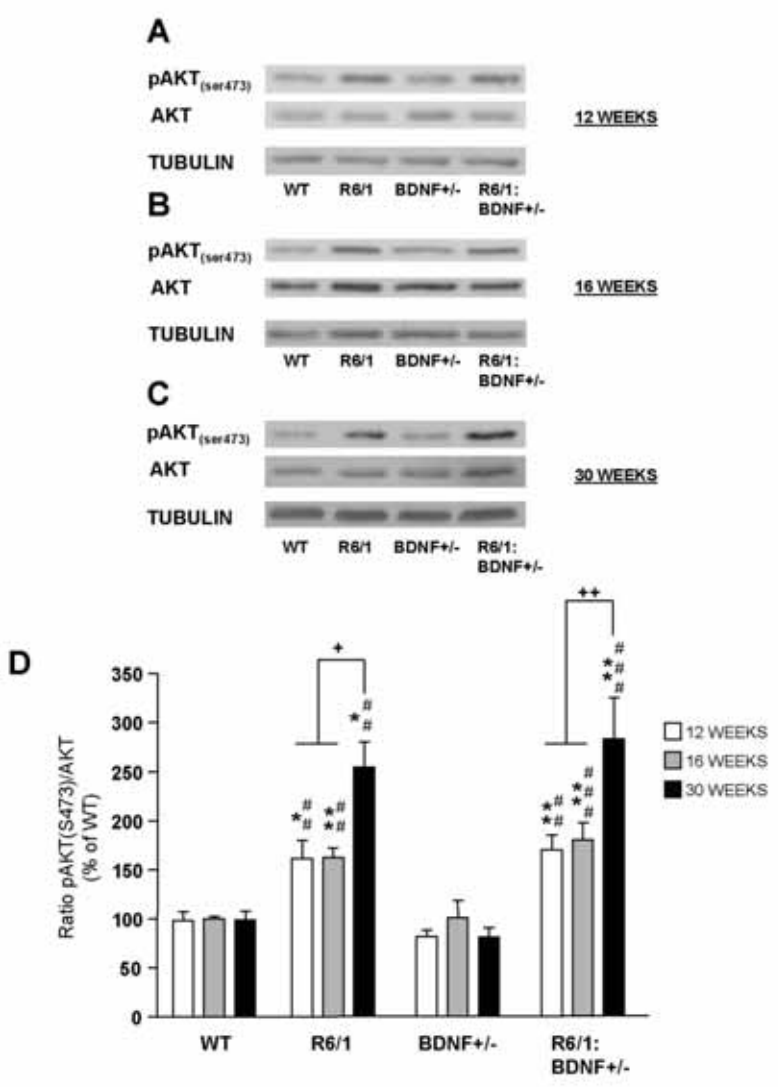

Fig. 1 AKT phosphorylation status is increased in the striatum early during the disease progression and maintained until late stages of neurodegeneration. Phospho(S473)-AKT levels were analyzed by Western blot in the striatum of R6/1 and R6/1:BDNF+/- mice at 12, 16 and 30 weeks of age. (A, B and C) Representative immunoblots showing protein levels of phospho(S473)-AKT, AKT and tubulin in wild-type (WT), R6/1, BDNF+/- and R6/1:BDNF+/- mice. (D) Figure showing the increase in phospho(S473)-AKT/AKT ratio in all the genotypes at different ages. Values are expressed as percentages of WT mice ± SEM for five animals per genotype. Data were analyzed by two-way ANOVA followed by Bonferroni's post-hoc test. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with WT mice; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ compared with BDNF +/- mice.; $^{+}P < 0.05$; $^{++}P < 0.01$ compared with 30 week-old animals.

Phospho(S473)-AKT levels are specifically increased in the striatum of R6/1 and R6/1:BDNF+/- mice

In order to confirm that the increase of phospho(S473)-AKT levels was not due to a general mechanism in all the regions of the HD mouse brain, we analyzed phosphorylated and total protein levels at 30 weeks in the cortex and hippocampus. In contrast to that observed in the striatum, no differences in phospho(S473)-AKT or total AKT levels were observed between R6/1 or R6/1:BDNF+/- and their littermate controls in either of the two areas (Fig. 2). These data show that in these HD mouse models the AKT pathway is specifically regulated in the striatum.

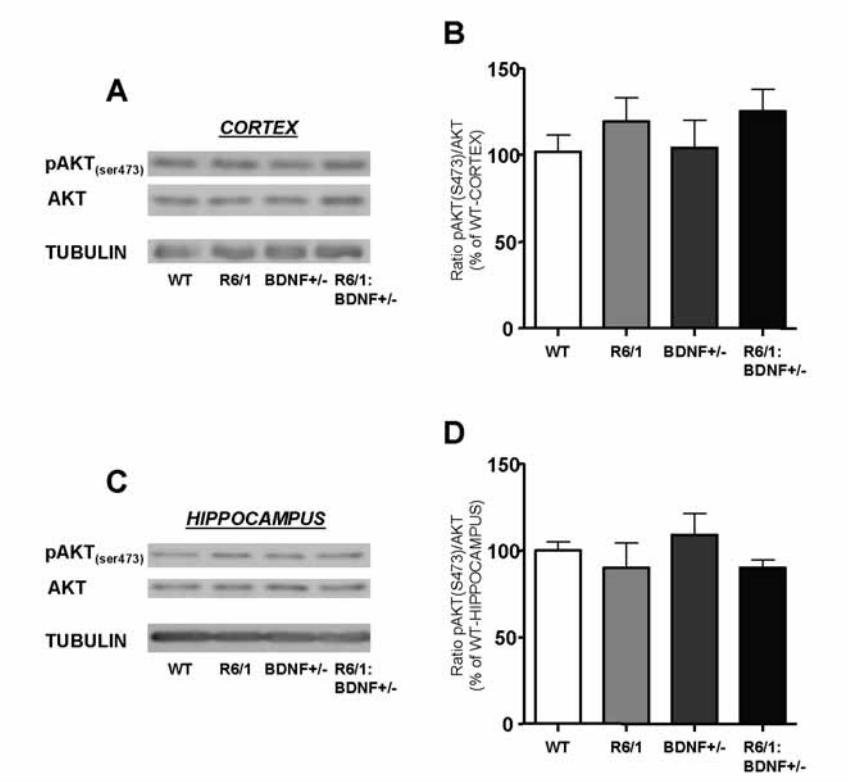

Fig. 2 No changes in AKT phosphorylation status in the cortex and hippocampus of HD mouse models. Western blot analysis of phospho(S473)-AKT in cortex and hippocampus of R6/1 and R6/1:BDNF+/- at 30 weeks of age. Immunoblots showing a representative experiment of phospho(S473)-AKT, AKT and tubulin protein levels in the cortex (A) and hippocampus (C) of wild-type (WT), R6/1, BDNF +/- and R6/1:BDNF+/- mice. Figures showing similar phospho(S473)-AKT/AKT ratio in the cortex (B) and hippocampus (D) of all the genotypes analyzed. Results are expressed as percentages of WT ± SEM for five animals per genotype. Data were analyzed by one-way ANOVA.

Increased AKT activation is associated with different prosurvival targets during disease progression

To test whether increased AKT activation was associated with altered AKT signaling in R6/1 and R6/1:BDNF+/- mouse striatum, we assessed phospho(S256)-FoxO1 and phospho(S9)-GSK3*β*, two different well-known downstream targets of the AKT pathway that are involved in neuronal survival.

At 16 weeks, the increase in AKT activity differentially affects the downstream targets. Consistent with AKT activation, the phospho(S9)-GSK3*β*/GSK3*β* ratio was significantly increased in both R6/1 and R6/1:BDNF+/- mice due to a large enhancement of phospho(S9)-GSK3*β* levels (Fig. 3A and 3B). However, at this age, no changes in phospho(S256)-FoxO1 or FoxO1 were detected in any of the genotypes analyzed (Fig. 3C and 3D).

We next analyzed protein levels at 30 weeks and, in contrast to 16-week observations, the increased AKT activation was accompanied by the enhancement of phosphorylation in both GSK3*β* and FoxO1 proteins. In 30 week-old striata we detected a large enhancement of phospho(S9)-GSK3*β* levels (Fig. 3E), which induces an increase in the phospho(S9)-GSK3*β*/GSK3*β* ratio in both R6/1 and R6/1:BDNF+/- mice (Fig. 3F). In addition, we also detected a significant increase of phospho(S256)-FoxO1 in HD mice (Fig. 3G) that resulted in the significant enhancement of the phospho(S256)-FoxO1/FoxO1 ratio (Fig. 3H), suggesting a more potent prosurvival activity of the kinase throughout disease progression. Interestingly, at this age we also observed a significant increase of FoxO1 total levels in both R6/1 and R6/1:BDNF+/- mice (Fig. 3G; wild-type: 100±6; BDNF+/-: 116±19; R6/1: 154±4$^{**\#}$; R6/1:BDNF+/-: 147±17$^{*\#}$; **P <0.01 and *P <0.05 compared with wild-type animals, #P <0.05 compared with

BDNF+/- animals), but this rise was not enough to modify the increase in the phosphorylated/total ratio.

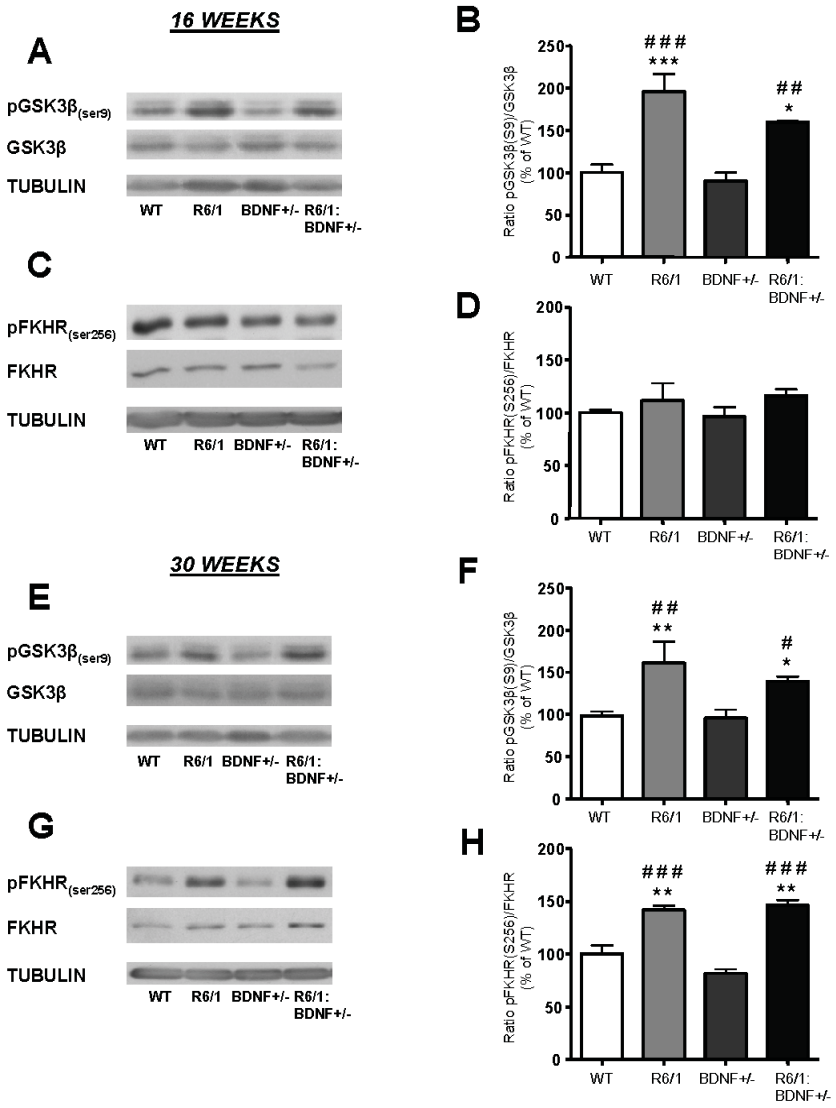

Fig. 3 Striatal levels of phospho-GSK3β and phospho-FoxO1 are differentially modified during disease progression. Levels of phospho(S9)-GSK3β and phospho(S256)-FoxO1 were analyzed by Western blot in the striatum of 16 and 30 week-old mice (n = 5 for each genotype). (A and C) Representative immunoblots showing phospho(S9)-GSK3β, GSK3β, phospho(S256)-FoxO1, FoxO1 and tubulin protein levels in striatal samples of wild-type (WT), R6/1, BDNF+/- and R6/1:BDNF+/- mice at 16 weeks. (E and G) Representative immunoblots showing phospho(S9)-GSK3β, GSK3β, phospho(S256)-FoxO1, FoxO1 and tubulin protein levels in striatal samples of wild-type (WT), R6/1, BDNF+/- and R6/1:BDNF+/- mice at 30 weeks. Figures showing phospho(S9)-GSK3β/ GSK3β (B and F) and phospho(S256)-FoxO1/FoxO1 (D and H) ratios in the striatum of 16 (B and D) and 30 week-old (F and H) animals. Immunoblots are representative of three different experiments. Values are expressed as percentages of WT mice ± SEM for five animals per genotype. Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ compared with WT mice; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ compared with BDNF +/- mice.

The R6/2 mouse model also displays striatal AKT activation at late stages of the neurodegenerative process

In order to find out if long-term AKT activation is a common feature of exon 1 mhtt mouse models, we next analyzed phosphorylated and total AKT levels in R6/2 mouse striatum. We assessed striatal AKT and phospho(S473)-AKT levels by western blot at 12 weeks, when R6/2 mouse is severely impaired. In accordance with the previous results observed at 30 weeks in R6/1 and R6/1:BDNF+/- mouse striatum, we observed a large increase of phospho(S473)-AKT levels in R6/2 striatum compared to littermate controls (Fig. 4A). This increase was accompanied by a significant enhancement of the phospho(S473)-AKT/AKT ratio (Fig. 4B). Interestingly, in spite of this increase in AKT activation status, total AKT levels were also increased within R6/2 striatum (Fig. 4A and 4C), pointing to more severe alterations of the pro-survival pathway in the R6/2 mouse.

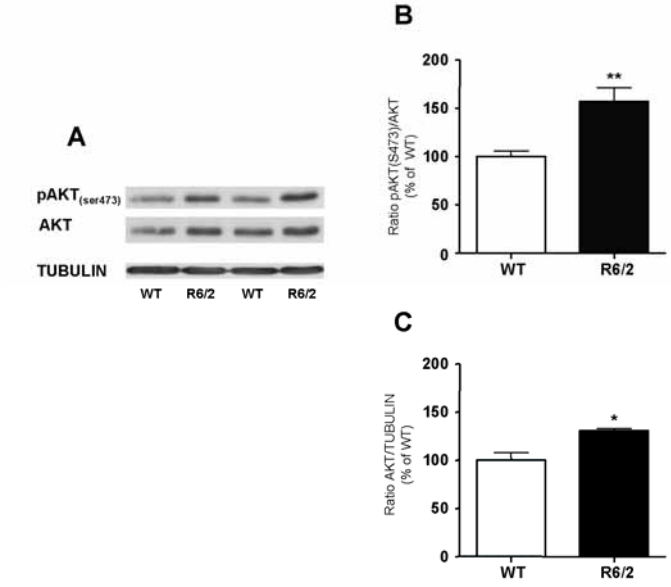

Fig. 4 Both phospho-AKT and AKT protein levels are increased in the striatum of R6/2 mouse. Phospho-AKT and AKT protein levels were analyzed by Western blot in 12 week-old R6/2 mice. (A) Representative immunoblots showing protein levels of phospho(S473)-AKT, AKT and tubulin of WT and R6/2 mice striatum. Figures showing phospho(S473)/AKT (B) and AKT/tubulin (C) ratio in the striatum of WT and R6/2 mice. Values are expressed as percentages of WT mice ± SEM for four animals per genotype. Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test. $*P < 0.05$; $**P < 0.01$ compared with WT mice.

Striatal AKT activation is partially reverted by suppressing transgene expression in Tet /HD94 mice striatum

To determine whether AKT activation depends on the stress induced by continuous mhtt expression, we analyzed its activation status in the Tet/HD94 mouse. Striatal AKT and phospho(S473)-AKT protein levels were examined at 22 months in three different groups: wild-type, Tet/HD94 with no pharmacological intervention (Gene-ON) and Tet/HD94 after 5 months of doxycycline administration that turns off transgene expression (Gene-OFF). In these conditions, the Gene-ON mice displayed severe striatal neuron loss compared to their control littermates (Diaz-Hernandez et al., 2005). Consistent with the results obtained in R6 models, we observed an increase in phospho(S473)-AKT levels in Tet/HD94 mouse striatum with respect to wild-type animals accompanied by the consequent enhancement of the phospho(S473)-AKT/AKT ratio (Fig. 5A and 5B).

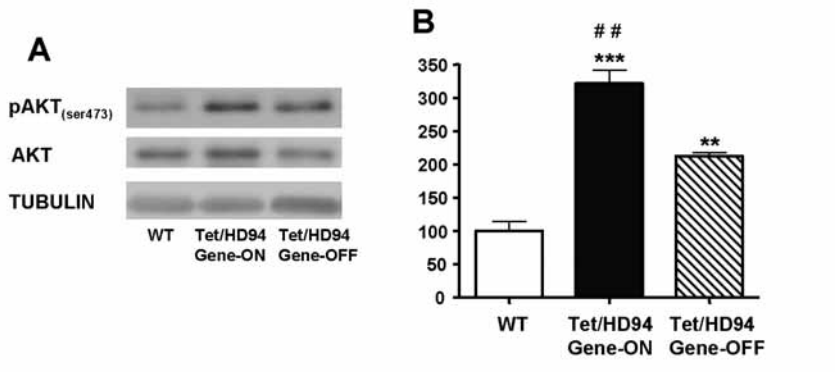

Fig. 5 Increased levels of phospho(S473)-AKT are partially reverted by suppressing transgene expression in the striatum of Tet/HD94 mice. Phospho-AKT and AKT protein levels were analyzed by Western blot in the striatum of wild-type (WT) and Tet/HD94 mice either with no pharmacological intervention (Gene-ON) or after 5 months of transgene shut-down by doxycycline administration (Gene-OFF). (A) Representative immunoblots showing protein levels of phospho(S473)-AKT, AKT and tubulin in the striatum of 22 month-old WT, Gene-ON and Gene-OFF mice. (B) Figures showing the densitometric measures of phospho(S473)-AKT normalized to AKT protein levels, and expressed as percentages of WT ± SEM for 4 animals per condition. Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with WT mice. $^{\#\#}P < 0.01$ compared with Tet/HD94 (Gene- OFF) mice.

Interestingly, by suppressing transgene expression the phospho(S473)-AKT/AKT ratio enlargement was partially reverted with a significant reduction of the phosphorylated levels (Fig. 5A and 5B), suggesting AKT activation as a prosurvival mechanism in response to the toxicity of mhtt expression.

Discussion

Our results show that phospho(Ser^{473})-AKT levels are specifically increased in the striatum of R6/1 and R6/1:BDNF+/- mice early during disease progression. Interestingly, we also show that this increase is stronger in late stages of the neurodegeneration, inducing a differential inactivation of the GSK3β and FoxO1 proteins throughout the neurodegenerative process. In addition, we studied AKT activation status in R6/2 and Tet/HD94 mice. According to our results in R6/1 and R6/1:BDNF+/- mice, the constitutive expression of the N-terminal exon 1 mhtt resulted in an increase of phospho(Ser^{473})-AKT levels within both R6/2 and Tet/HD94 striatum. Moreover, we show that this increase was partially reverted in the latter after transgene shut-down, pointing to a pro-survival mechanism activated in response to N-terminal exon 1 mhtt-induced toxicity.

In this study, we demonstrated that phospho(S473)-AKT levels are significantly increased in the striatum of both R6/1 and R6/1:BDNF+/- mice and that this increase can be detected early during disease progression (12 and 16 weeks). Interestingly, we show that this pro-survival activity can still be detected at late stages of the neurodegenerative process (30 weeks), and furthermore, this AKT phosphorylation is higher than at earlier stages. This increase of phospho(Ser^{473})-AKT in the striatum seems to be a common feature for mutant htt exon-1 mouse models, as it can also be detected in 12 week-old R6/2 mouse striatum. We also show that increased levels of

phosphorylated AKT were not due to a general mechanism within the brain, since no changes in the activation status of AKT in the cortex and hippocampus were detected. This would explain why other studies have failed to find changes in phospho-AKT levels in R6/2 mice using whole brain lysates (Warby et al., 2005). Conversely, recent data obtained from an acute *in vivo* rat model consisting on intra-striatal lentiviral injections of N-terminal fragments of htt with a poly-Q stretch of 19 or 82 residues, demonstrate that both AKT and phospho-AKT levels are progressively down-regulated during neuronal dysfunction (Colin et al., 2005). These authors have also shown that AKT is proteolytically processed in the brain tissues of grade 3 and 4 HD patients, leading to its irreversible deactivation. Altogether, with the early increase of phospho(Ser^{473})-AKT in R6/1 and R6/1:BDNF+/- mice, our data support the hypothesis of a sequential process affecting the AKT pathway in HD (Colin et al., 2005). Interestingly, we showed that these mouse models, which display weak or no loss of striatal cells, maintain a large increase in the AKT phosphorylation status, in contrast to the lentiviral model that displays reduced levels of both AKT and phospho-AKT levels. Thus, pointing to the loss of AKT activity as a crucial event that induces massive striatal cell death in HD.

At earlier stages (16 weeks), AKT activation is coupled to prosurvival signaling via phospho(S9)-GSK3β enhancement. In accordance with our results, previous studies have shown increased levels of phospho-AKT as an early prosurvival response in ST$Hdh^{Q111/Q111}$ striatal cell line *in vitro*, and this prosurvival signaling is mediated, at least in part, via GSK3β inactivation (Gines et al., 2003). At late stages (30 weeks), we observed that AKT activation was still associated with prosurvival signaling via GSK3β inactivation and, furthermore, the protein kinase exerts a more extensive effect by blocking the pro-apoptotic protein FoxO1. Thus, the effect of the AKT activation in

these HD mouse models is associated with the prosurvival signaling via GSK3*β* inactivation during the evolution of the neuropathological process, while the effect on the pro-apoptotic target FoxO1 seems to be specific to the late stages of pathology. Different lines of studies also support the neuroprotective role of AKT pathway in HD (Humbert et al., 2002). These authors demonstrated htt phosphorylation at S421 by AKT and suggested that this direct action on mhtt, together with phosphorylation of other substrates, increases neuronal survival. We have to consider that all HD models expressing the N-terminal exon 1 fragment, lack the S421 AKT phosphorylation site and, therefore, this fragment cannot be phosphorylated. Therefore, the prosurvival effect exerted by AKT over R6 striatal neurons must be mediated through the inhibition of apoptotic pathways activated in response to N-terminal exon 1 mhtt toxicity, such as GSK3*β* and FoxO1. In addition, we have recently described that, instead of displaying a mild apoptotic response, R6/1 and R6/1:BDNF+/- mice exhibit a high increase of the pro-apoptotic proteins Bid and Bim in the mitochondrial fraction together with a slight accumulation of the pro-apoptotic effector Bax (Garcia-Martinez et al., 2007). Therefore, an alternative mechanism of AKT activity could be the inhibition of a massive Bax recruitment to the mitochondria, blocking the evolution of the apoptotic progress as previously described (Yamaguchi and Wang, 2001).

We also analyzed AKT activation status in a conditional mouse model of HD, the Tet/HD94 mouse. At 17 months of age, Tet/HD94 mice show deficits in motor coordination, a decrease in striatal volume and a significant decrease in the number of striatal neurons that progress with age (Diaz-Hernandez et al., 2005). Here we show that, similar to that observed in R6/1 and R6/1:BDNF+/- mice, Tet/HD94 mice displayed increased levels of phospho(Ser^{473})-AKT protein in the striatum at 22 months of age. Interestingly, we observed that transgene shut-down during 5 months partially

reverted phosphorylated AKT up-regulation. Thus, supporting the hypothesis that the phospho-AKT increase is directly related with the toxic effect mediated by mhtt expression. Despite 19% of striatal cell loss, after 5 months of doxycycline treatment Tet/HD94 mice display full recovery from their motor deficit (Diaz-Hernandez et al., 2005), and the authors proposed that the remaining 80% of striatal neurons are able to recover their function to a level that allows them to compensate for missing neurons. These data further support the importance of maintaining increased phospho-AKT levels, which can act against N-terminal exon 1 mhtt toxicity, delaying irreversible cell loss and allowing the recovery of neuronal functionality after transgene shut-down.

One of the most important mechanisms of AKT activation is the intracellular signaling mediated by trophic factors (Burgering and Coffer 1995; Franke et al., 1995). Among these, the neurotrophic factor BDNF displays a predominant role in promoting striatal neuron survival both *in vitro* (Ventimiglia et al., 1995; Gavalda et al., 2004) and *in vivo* (Perez-Navarro et al 1999, 2000, 2005; Gratacos et al., 2001). Furthermore, a deficit in this neurotrophin has been proposed as one of the mechanisms that can participate in the striatal neuron dysfunction induced by mutant htt (Zuccato et al., 2001; 2003; Gauthier et al., 2004; Canals et al., 2004). However, the activation of AKT in R6 models seems to be independent of endogenous BDNF levels, since R6/1:BDNF+/- and R6/2 mice with lower BDNF expression display higher levels of phospho(Ser^{473})-AKT compared to their littermate controls. Similarly, no differences were detected between R6/1 and R6/1:BDNF+/- mice at any of the time points analyzed. Therefore, a different mechanism must be involved in the specific increase of phospho(Ser^{473})-AKT in mouse striatum. Previous work with Q111/Q111 striatal cell line *in vitro* have shown that AKT activation in mutant striatal cells can be blocked by the addition of the N-methyl-D-Aspartate (NMDA) receptor antagonist MK-801 and,

moreover, this activation is calcium (Ca^{2+}) dependent (Gines et al., 2003). Interestingly, different HD mouse models display an enhancement of currents induced by selective activation of NMDA receptors, as well as an enhancement of intracellular Ca^{2+} flux in both pre-symptomatic and symptomatic stages (Cepeda et al., 2001). Another possibility is that AKT remains activated in response to other stress stimulus. Different chaperones induced by cellular stress, such as Hsp27 and Hsp90, could specifically bind phospho–AKT blocking its degradation (Konishi et al., 1997; Rane et al., 2003). Moreover, the activation of AKT in response to oxidative stress, a well-known feature of HD (Rego and Olivera, 2003), has also been described in cardiomyocytes (Tu et al., 2002). Altogether, although AKT prosurvival activation could reflect an enhancement of NMDA receptor signaling, similar to the knockin model, the possible participation of different mechanisms in response to intracellular stress in maintaining the anti-apoptotic signaling must also be considered.

In summary, in this work, we show that phospho-AKT levels are up-regulated in four different HD mouse models that express N-terminal exon 1 of mhtt and, moreover, that this AKT activation is directly associated with the inhibition of GSK3β and FOXO pro-apoptotic pathways. We also show that this increase can still be detected at late stages of the neurodegenerative process and can contribute to the modest neuronal death described in these mice. These data further support the crucial relevance of this prosurvival pathway and its use in combined therapeutic approaches as an effective means of delaying striatal cell death in HD.

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