Cuarto trabajo

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Brain-derived neurotrophic factor prevents changes in Bcl-2 family members and caspase-3 activation induced by excitotoxicity in the striatum

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Abstract

Brain-derived neurotrophic factor (BDNF) prevents the loss of striatal neurons caused by excitotoxicity. We examined whether these neuroprotective effects are mediated by changes in the regulation of Bcl-2 family members. We first analyzed the involvement of the phosphatidylinositol 3-kinase/Akt pathway in this regulation, showing a reduction in phosphorylated Akt (p-Akt) levels after both quinolinate (QUIN, an NMDA receptor agonist) and kainate (KA, a non-NMDA receptor agonist) intrastriatal injection. Our results also show that Bcl-2, Bcl-x_L and Bax protein levels and heterodimerization are selectively regulated by NMDA and non-NMDA receptor stimulation. Striatal cell death induced by QUIN is mediated by an increase in Bax and a decrease in Bcl-2 protein levels, leading to reduced levels of Bax:Bcl-2 heterodimers. In contrast, changes in Bax protein levels are not required for KA-induced apoptotic cell death, but decreased levels of both Bax:Bcl-2 and Bax:Bcl-x_L heterodimer levels are necessary. Furthermore, QUIN and KA injection activated caspase-3. Intrastriatal grafting of a BDNF-secreting cell line counter-regulated p-AKT, Bcl-2, Bcl-x_L and Bax protein levels, prevented changes in the heterodimerization between Bax and pro-survival proteins, and blocked caspase-3 activation induced by excitotoxicity. These results provide a possible mechanism to explain the anti-apoptotic effect of BDNF against to excitotoxicity in the striatum through the regulation of Bcl-2 family members, which is probably mediated by Akt activation.

Keywords: apoptosis, basal ganglia, Bax knock-out, kainate, neurotrophic, quinolinate.

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Prolonged activation of glutamate receptors leads to neuronal death by excitotoxicity. In the central nervous system, this type of cell death has been associated with the development of chronic neurodegenerative disorders, such as Parkinson's disease and Huntington's chorea (Choi 1988; Alexi *et al.* 2000). Glutamate receptor overstimulation activates both necrotic and apoptotic pathways (Ferrer *et al.* 1995; Qin *et al.* 1996; Tenneti *et al.* 1998). In the past years, extensive research has been devoted to understanding the regulation of apoptosis as a potential route to the prevention of cell death in disease conditions. Indeed, apoptosis is mediated and regulated by intrinsic factors such as the Bcl-2 family (Cory and Adams 2002), and mechanisms such as mitochondrial release of cytochrome *c* (Li *et al.* 1997), and the activation of caspases (Yuan *et al.* 1993).

The Bcl-2 family comprises at least 12 related proteins that can be divided into two groups according to functional criteria. Members of the first group, such as Bcl-2 and Bcl-x_L, exhibit anti-apoptotic activity, whereas proteins of the second group exert pro-apoptotic effects, Bax being the prototypical member. These proteins can undergo various modifications in response to an apoptotic stimulus, including phosphorylation

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Abbreviations used: Ac-DEVD-acf, acetyl-Asp[OMe]-Glu[OMe]-Val-Asp[OMe]-7-amino-4-trifluoromethyl coumarin; AP, anteroposterior; BCIP, 5-Bromo-4-chloro-3-indolyl-phosphate; BDNF, brain-derived neurotrophic factor; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione disodium; ECL, enhanced chemiluminiscence; KA, kainate; MK-801, dizocilpine maleate; ML, mediolateral; NBT, 4-nitro blue tetrazolium chloride; PBS, phosphate buffered saline; p-Akt, phosphorylated Akt; PI3K, phosphatidylinositol 3-kinase; QUIN, quinolinate; TBS-T, Tris-buffered saline with Tween; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling.

and proteolysis, and changes in conformation, intracellular localization and expression. Some of these modifications allow the association of these proteins as homodimers or heterodimers. In this context, Bcl-2 and Bcl-x_L can inhibit pro-apoptotic members of the Bcl-2 family through heterodimerization, whereas Bax homodimerization activates cell death (for review see Antonsson 2001; Cory and Adams 2002).

The Bcl-2 family participates in the regulation of programmed cell death during development and in the apoptotic process induced by a wide array of cytotoxic insults. Consistent with this, Bax knock-out mice are more resistant to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dopaminergic degeneration (Vila *et al.* 2001), as are transgenic mice overexpressing Bcl-2 (Offen *et al.* 1998). Moreover, in several models of neuronal injury the expression of either Bcl-2 or Bcl-x_L is decreased (Krajewski *et al.* 1995; Sato *et al.* 1998; Tamatani *et al.* 1998; Ghribi *et al.* 2002; Wei *et al.* 2002), whereas Bax expression is up-regulated (Krajewski *et al.* 1995; Ghribi *et al.* 2002). The Bcl-2 protein family is also involved in cell death induced by excitotoxicity. For instance, induction of Bcl-2 expression from a viral vector, delivered either before or after the insult, protects cortical neurons from glutamate excitotoxicity (Jia *et al.* 1996). Furthermore, overexpression of Bcl-2 protects cultured cortical neurons from apoptosis induced by stimulation of the AMPA glutamate receptor (Cheung *et al.* 2000).

Several neuroprotective agents, such as brain-derived neurotrophic factor (BDNF), exert neuroprotective effects by regulating Bcl-2 family members (Tamatani *et al.* 1998; Chen and Chuang 1999; Sawada *et al.* 2000; Schabitz *et al.* 2000; Ghribi *et al.* 2002). One of the signaling cascades activated by binding of BDNF to its receptor, TrkB, is the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Patapoutian and Reichardt 2001; Chao 2003). The activation of this pathway has been implicated in neuronal survival (Dudek *et al.* 1997; Hetman *et al.* 1999; Vaillant *et al.* 1999; Gavalda *et al.* 2004) and regulates Bcl-2 family members (Datta *et al.* 1997; Skorski *et al.* 1997; Riccio *et al.* 1999). As BDNF protects striatal neurons against excitotoxicity induced by stimulation of NMDA (Perez-Navarro *et al.* 1999, 2000a) and non-NMDA receptors (Gratacos *et al.* 2001), we aimed to determine whether the Bcl-2 protein family is involved in these neuroprotective effects. To this end, we analyzed changes in the protein levels of phosphorylated Akt (p-Akt), Bcl-2, Bcl-x_L and Bax after intrastriatal injection of quinolinate (QUIN, an NMDA receptor agonist) or kainate (KA, a non-NMDA receptor agonist). Furthermore, the heterodimerization between Bcl-2 family members, alongside an assessment of caspase-3 activation was also examined. To assess the effects of BDNF on these changes, a fibroblast cell line secreting high levels of the neurotrophin was grafted into the striatum before QUIN or KA injection.

Materials and methods

Materials

Adult male Fischer-344 rats and heterozygous Bax mice were purchased from Harlan Interfauna (Spain) and Jackson Laboratories (Bar Harbor, ME, USA), respectively. QUIN, KA, 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX), dizocilpine maleate [(+)-MK-801], and bovine serum albumin were obtained from Sigma Chemical Co. (St Louis, MO, USA). Bax primers were from Qiagen (Hilden, Germany). Apoptosis detection system (fluorescein) and horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were obtained from Promega (Madison, WI, USA). Fluoro-Jade was from Histo-Chem Inc. (Jefferson, AR, USA). For western blot analysis, Bcl-2 and Bax antibodies were from Pharmingen (San Diego, CA, USA), Bcl-x_L and pan-ERK antibodies were from Transduction Laboratories (Lexington, KY, USA), activated serine/threonine kinase Akt and pan-Akt were from Cell Signaling Technology (Beverly, MA), and polyvinylidene difluoride membranes (Immobilon-P) were from Millipore (Massachusetts, MA, USA). Monoclonal Bcl-2 antibody used for immunoprecipitation was obtained from Transduction Laboratories. 4-Nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were obtained from Roche (Basel, Switzerland). Enhanced chemiluminescence (ECL) and Protein A sepharose were from Amersham Biosciences (Freiburg, Germany). The fluorogenic substrate acetyl-Asp[OMe]-Glu[OMe]-Val-Asp[OMe]-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-acf) was from Enzyme Systems Products (Livermore, CA, USA). The Avidin-Biotin Complex (ABC) kit was from Pierce (Tattenhall, UK). Phoretix 1D Gel analysis software was from Phoretix International Ltd. (Newcastle, UK).

Animal subjects

Adult male Fischer-344 rats (200–250 g) and Bax-deficient transgenic mice (8 weeks old) were used in this study. Heterozygous Bax mice were bred to maintain the colony and to obtain Bax –/–,+/–, and wild-type genotypes. At postnatal day 21, tail DNA was prepared and screened for both the normal and the mutant allele by single PCR in accordance with the protocol described by Deckwerth *et al.* (1996), with minor modifications. The primers used were the following: Bax intron 5 reverse primer, 5′-GGTGACCAGAGTGGCGTAGG-3′; Neo/PGK reverse primer, 5′-CCGCTTCCATTGCTCAGCGG-3′; Bax exon 5 forward primer, 5′-GAGCTGATCAGAACCATCATG-3′. Cycling parameters were: 1 min at 94°C, 2 min at 55°C and 3 min at 72°C each for a total of 30 cycles. PCR products were resolved on 2% agarose gels.

After surgery, animals were housed separately with food and water *ad libitum* in a colony room maintained at a constant temperature (19–22°C) and humidity (40–50%) on a 12 : 12 h light/dark cycle. Animal treatments and handling procedures were approved by the Local Committee (99/1 University of Barcelona) and the Generalitat de Catalunya (1094/99), in accordance with the European Communities Council Directive (86/609/EU).

Excitotoxic lesion and cell grafting

Rats were anesthetized with pentobarbital and two microinjections of QUIN (34 nmol or 68 nmol), KA (0.5 nmol or 1 nmol) or vehicle (phosphate-buffered saline) were performed. In another set of experiments, QUIN and KA were coinjected with glutamate receptor antagonists at the following doses: MK-801, 15 nmol and CNQX, 0.5 nmol. The anteroposterior (AP) and mediolateral (ML) coordinates relative to bregma were (i) AP +2.2 mm, ML +2.9 mm and (ii) AP +0.8 mm, ML +3.5 mm. To study the effects of BDNF on changes induced in Bcl-2 family members by excitotoxicity, Fischer 344 rat 3T3 fibroblasts transfected with a BDNF cDNA (F3N-BDNF; Neveu and Arenas 1996) or mock-transfected Fischer 344 rat 3T3 fibroblasts (F3A-MT, control; Arenas and Persson 1994) were intrastriatally grafted as described elsewhere (Perez-Navarro *et al.* 1999, 2000a). We have previously shown that 8 days after intrastriatal grafting of the F3N-BDNF cell line, BDNF content was increased by 17-fold compared with striata receiving the control graft (Perez-Navarro *et al.* 2000a). Cells (7.5×10^5 in 3 μL) were stereotaxically injected into the striatum (1 μL/min) at the following coordinates: AP +1.8 mm, ML +3.2 mm. Twenty-four hours later, animals were injected with phosphate-buffered saline, QUIN or KA using the same coordinates as described above. In all cases, the injection was performed at 5.2 mm below the dural surface with the incisor bar at 5 mm above the interaural line.

Wild-type and Bax knock-out mice were anesthetized with pentobarbital and an intrastriatal microinjection of phosphate-buffered saline, QUIN (12 nmol) or KA (0.5 nmol) was performed 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.

In situ detection of DNA fragmentation

Forty-eight hours after intrastriatal QUIN or KA injection, rats ($n = 3$ animals for each condition) were deeply anaesthetized and immediately perfused transcardially with phosphate-buffered saline, followed by 4% paraformaldehyde/phosphate buffer (0.1 M, pH 7.4). Brains were removed and post-fixed for 1–2 h in the same solution, cryoprotected by immersion in 15% sucrose/phosphate-buffered saline and then frozen in dry-ice-cooled isopentane. Horizontal cryostat sections (14 μm) through the whole striatum were serially collected on silane-coated slides. DNA fragmentation was histologically examined using an *in situ* Apoptosis detection system and performed as described elsewhere (Perez-Navarro *et al.* 2000a).

Western blot analysis

Protein levels of p-Akt, Bcl-2, Bcl-x$_L$ and Bax were examined by western blot at various time points after phosphate-buffered saline, QUIN or KA intrastriatal injection in non-grafted striata ($n = 4$ for each condition and time point). These proteins were then analyzed in animals injected with QUIN plus MK-801 or KA plus CNQX, and in animals grafted with control or BDNF-secreting cell lines prior to injection with phosphate-buffered saline, QUIN or KA ($n = 4$ for each condition). Protein extracts obtained from frozen striata (30 μg) were denatured at 100°C for 5 min in sample buffer and loaded on a 15% denaturing polyacrylamide gel using the Mini-protean system. Proteins were then transferred to a polyvinylidene difluoride membrane washed twice in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and incubated for 1 h with 5% bovine serum albumin and 5% skimmed milk in TBS-T. Membranes were then incubated overnight at 4°C with antibodies against p-Akt (rabbit polyclonal, 1: 2000), Bcl-2 (rabbit polyclonal, 1: 2000), Bcl-x$_L$ (mouse monoclonal, 1 : 500) or Bax (rabbit polyclonal, 1 : 1500). On the following day, after two rinses with TBS-T, membranes were incubated for 1 h with anti-rabbit or anti-mouse Ig linked secondary antibodies (1 : 1000), and the reaction was finally visualized using a chemiluminescence detection system (ECL). To make sure that protein was equally loaded in each lane, membranes were incubated for 1 h at room temperature (22°C) with a mouse monoclonal antibody against pan-ERK (1 : 5000) or against pan-Akt (1 : 2000). After two rinses with TBS-T, membranes were incubated for 1 h at room temperature with anti-mouse Ig alkaline phosphatase-conjugated secondary antibodies (1 : 10 000). The reaction was visualized by incubation with NBT and BCIP. Western blot replicates were scanned and quantified using Phoretix 1D Gel Analysis software.

Immunoprecipitation

Protein (250 μg) obtained from frozen striata ($n = 3$–5 for each condition) was incubated overnight at 4°C on a rotary mixer with anti-Bax polyclonal antibody (1 : 100) diluted in lysis buffer (50 mM Tris pH 7.5, 10% glycerol, 1% Triton X-100, 150 mM NaCl, 100 mM NaF, 5 μM $ZnCl_2$, 1 mM Na_3VO_4, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin and 1 μg/mL leupeptin). The immune complexes were precipitated overnight at 4°C with the addition of 30 μL of 5% (w/v) protein A Sepharose, pre-blocked with 10% bovine serum albumin for 1 h. Beads were collected by centrifugation and washed three times with Tris-Triton buffer (20 mM Tris pH 7.5, 150 mM NaCl and 0.1% Triton X-100). Blots were immunostained with either a monoclonal antibody against Bcl-2 (1 : 500) or a monoclonal antibody against Bcl-x$_L$ (1 : 500). To ensure that Bax was correctly immunoprecipitated, all membranes were incubated overnight at 4°C with anti-Bax antibody (1 : 1500). After two rinses with TBS-T, membranes were incubated for 1 h at room temperature with anti-rabbit Ig alkaline phosphatase-conjugated secondary antibodies. The reaction was visualized by incubation with NBT and BCIP.

DEVD cleavage assay

Rats injected with phosphate-buffered saline, QUIN or KA were killed after 24 or 48 h ($n = 4$ for each condition and time point). Striata were quickly dissected out and frozen on dry ice. Proteins from frozen striata were prepared by homogenization in 500 μL lysis buffer (100 mM Hepes/NaOH pH 7.4, 150 mM NaCl, 5 mM dithiothreitol, 5 mM EDTA, 1% NP-40 and 20% glycerol) and centrifugation at 17 000 *g* for 15 min. Protein (20 μg) was incubated in a 96-well plate with the lysis buffer plus 20 μM of the fluorogenic substrate Ac-DEVD-acf. Plates were incubated at 37°C for 30 min and the increase in fluorescence was monitored (excitation at 360 nm and emission at 460 nm) using a fluorescence spectrophotometer. The Ac-DEVD-acf cleavage assay was also performed on striata grafted with control or BDNF-secreting cell lines plus phosphate-buffered saline, QUIN or KA. These animals were killed at different time points, according to the results obtained in non-grafted animals. The time points chosen were 24 h after KA and 48 h after QUIN intrastriatal injection ($n = 3$ for each condition).

Immunohistochemistry

For immunohistochemical analysis, animals ($n = 3$–5 for each condition) were deeply anesthetized and immediately perfused transcardially with saline followed by 4% paraformaldehyde/phosphate buffer. Brains were removed and post-fixed for 1–2 h in the same solution, cryoprotected by immersion in 30% sucrose/phosphate-buffered saline and then frozen in dry ice-cooled isopentane. Serial horizontal cryostat sections (40 μm) through the whole striatum were collected in phosphate-buffered saline as free-floating sections and stained with Nissl, anti-Bcl-2 (1 : 500), anti-Bcl-x_L (1 : 500) or anti-Bax (1 : 1000) antibodies. After treatment with H_2O_2 (0.3% in phosphate-buffered saline, 10% methanol) for 15 min and blocking with 5% normal horse serum and 0.2% bovine serum albumin for 2 h, sections were incubated with primary antibodies for 16 h (anti-Bcl-x_L and anti-Bax) or 48 h (anti-Bcl-2) at 4°C. After washing, they were incubated with biotinylated secondary antibodies (ABC kit, 1 : 200) and then with avidin–biotin complex. Finally, sections were developed with 0.05% diaminobenzidine, 0.01% $NiCl_2$ and 0.02% H_2O_2. As negative controls, some sections were processed as described above in the absence of primary antibody. Cells showing a positive signal for Bcl-2 and Nissl-stained neurons were counted in a region close to the injection site, as described previously (Perez-Navarro *et al.* 2000a; Gratacos *et al.* 2001).

Fluoro-Jade staining

Wild-type and Bax null mutant mice were deeply anesthetized and immediately perfused transcardially with saline followed by 4% paraformaldehyde/phosphate buffer, 48 h after phosphate-buffered saline, QUIN or KA intrastriatal injection ($n = 4$ for each condition). Brains were removed and post-fixed for 1–2 h in the same solution, cryoprotected by immersion in 30% sucrose/phosphate-buffered saline and then frozen in dry ice-cooled isopentane. Serial horizontal cryostat sections (20 μm) through the whole striatum were collected on silane-coated slides. Striatal sections were processed for Fluoro-Jade staining as described elsewhere (Schmued *et al.* 1997). Sections stained with Fluoro-Jade were visualized on a computer and the border of the lesion was outlined. The volume of the lesion was estimated by multiplying the sum of all the sectional areas (μm^2) by the distance between successive sections (180 μm), as described previously (Perez-Navarro *et al.* 2000a).

Results

Quinolinate and kainate intrastriatal injection induce morphologically similar patterns of cell death

Cell death induced by intrastriatal QUIN (34 nmol) or KA (0.5 nmol) injection was examined using the TUNEL (terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling) technique. Based on previous studies, we performed this analysis at 48 h because the number of TUNEL-labeled cells peaks between 48 and 72 h post-intrastriatal QUIN injection (Hughes *et al.* 1996). Our results show a morphologically similar pattern of cell death induced by both QUIN and KA (Figs 1b and c). TUNEL-positive nuclei were localized in lesioned striata, whereas almost no labeling was observed in phosphate-buffered saline-injected striata (Fig. 1a) or in other regions of the brain. These nuclei appeared condensed when observed at low magnification (Fig. 1). At higher magnification, many dying cells showed apoptotic morphology, bearing fragmented nuclei (insets in Figs 1b and c).

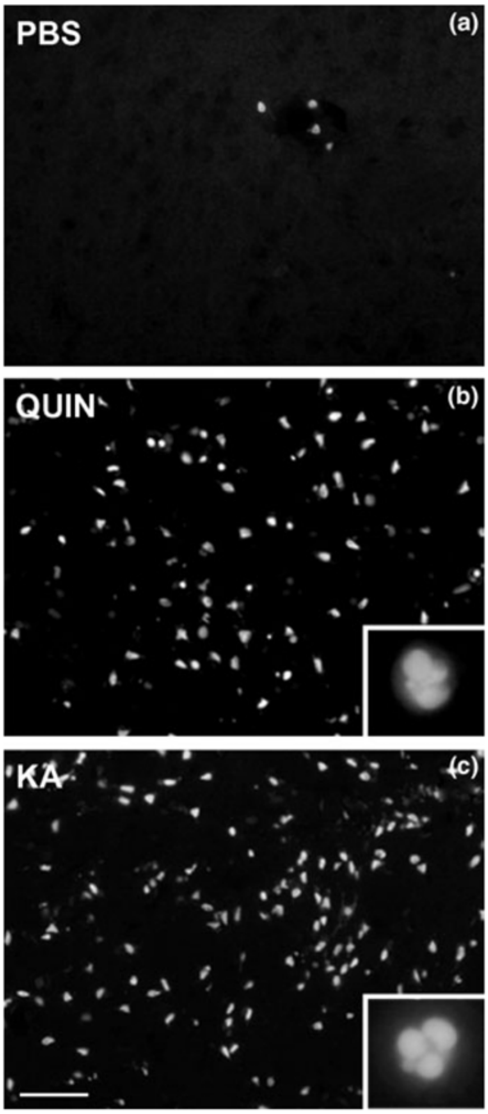


Fig. 1 Quinolinate (QUIN) and kainate (KA) injection induce apoptotic cell death in the striatum. Striata injected with QUIN or KA were examined for DNA fragmentation using the TUNEL (terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling) technique. Photomicrographs show TUNEL-labeled nuclei in a region close to the injection site 48 h after intrastriatal injection of phosphate-buffered saline (PBS) (a), 34 nmol QUIN (b) or 0.5 nmol KA (c). Insets in (b) and (c) show high-power images of a single TUNEL-labeled striatal neuron bearing nuclear DNA fragments. Scale bar indicates 50 μm for the lower magnification and 3 μm for the insets.

Quinolinate- and kainate-induced reduction in p-Akt protein levels is prevented by brain-derived neurotrophic factor

To begin exploring intracellular pathways mediating QUIN- and KA-induced cell death we examined p-Akt protein levels, which are regulated by BDNF (Gavalda *et al*. 2004). Intrastriatal QUIN (Fig. 2a) and KA (Fig. 2b) injection similarly induced a transient reduction, from 24 h to 48 h, in p-Akt protein levels. To test whether BDNF can regulate changes in p-Akt levels induced by excitotoxicity we used experimental conditions under which BDNF is neuroprotective (Perez-Navarro *et al*. 1999, 2000a; Gratacos *et al*. 2001). Intrastriatal grafting of a BDNF-secreting cell line blocked the decrease in p-Akt protein levels induced by QUIN (Fig. 2a) and KA (Fig. 2b), 48 h after injection. Furthermore, glutamate receptor antagonists, MK-801 (a specific NMDA receptor blocker) and CNQX (a specific non-NMDA receptor antagonist) prevented the reduction in p-Akt protein levels induced by QUIN (Fig. 2a) and KA (Fig. 2b), respectively. Total Akt levels were not altered by any of the above treatments (Fig. 2).

Bcl-2 and Bcl-x_L protein levels are differentially regulated by quinolinate and kainate

Intrastriatal QUIN or KA injection, at all doses tested, similarly regulated Bcl-2 protein levels: they showed an increase at 24 h and a marked decrease 48 h after injection (Figs 3a and c). In contrast, Bcl-x_L protein levels were differentially regulated by NMDA and non-NMDA receptors

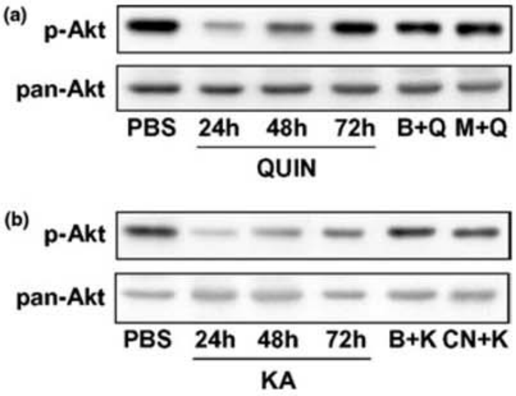
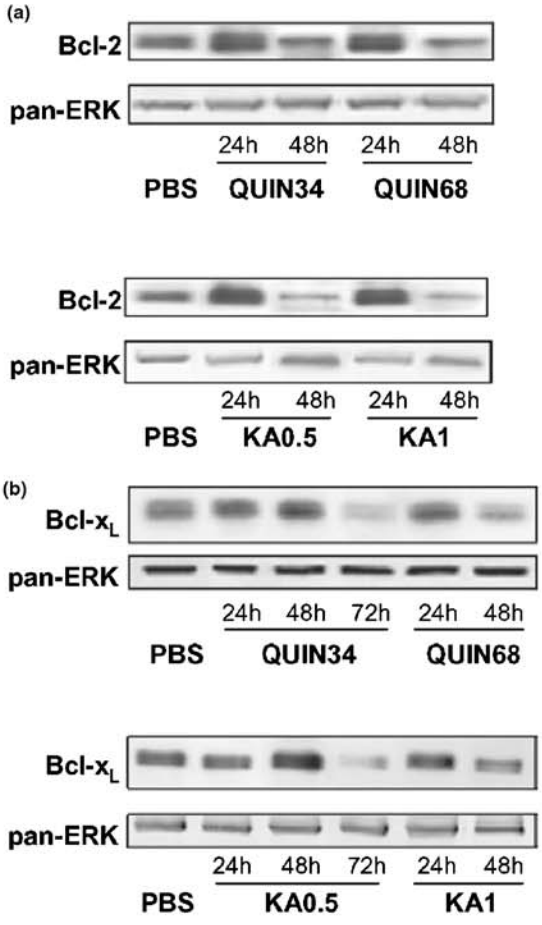


Fig. 2 Regulation of phosphorylated-Akt (p-Akt) protein levels by glutamate receptor stimulation and brain-derived neurotrophic factor (BDNF). The protein levels of p-Akt were examined by western blot. (a) Representative immunoblot showing p-Akt protein levels at different time points after 34 nmol quinolinate (QUIN) intrastriatal injection, 48 h after QUIN injection in striata grafted with BDNF secreting cells (B + Q), and 48 h after coinjection of QUIN plus MK-801 (M + Q). (b) Representative immunoblot showing p-Akt protein levels at different time points after intrastriatal injection of 0.5 nmol kainate (KA), 48 h after KA injection in striata grafted with BDNF secreting cells (B + K), and 48 h after KA plus 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX) injection (CN + K).



(c)

	Bcl-2		Bcl-x_L		
	24 h	48h	24 h	48h	72h
QUIN 34 nmol	178±11**	28±3**	93±10	105±32	18±5**
QUIN 68 nmol	240±22**	20±5**	117±7	39±9**	N.D.
KA 0.5 nmol	244±32**	10±2**	114±12	156±18*	14±2**
KA 1 nmol	388±80**	8±1**	152±18*	65±7*	N.D.

Fig. 3 Differential effects of quinolinate (QUIN) and kainate (KA) intrastriatal injection on Bcl-2 and Bcl-x_L protein expression. Bcl-2 (a) and Bcl-x_L (b) protein levels were analyzed by western blot at various time points after intrastriatal injection of different doses of QUIN (34 nmol, QUIN34; 68 nmol, QUIN68) or KA (0.5 nmol, KA0.5; 1 nmol, KA1). Immunoblots were obtained from representative experiments. (c) Table showing results obtained from densitometric measures expressed as percentages of control (phosphate-buffered saline-injected striata) ± SEM for four animals per condition. $^{**}p < 0.01$; $^{*}p < 0.05$, compared with control values. Statistical analysis performed by one-way analysis of variance (ANOVA) followed by the Scheffé *post hoc* test. N.D., not determined.

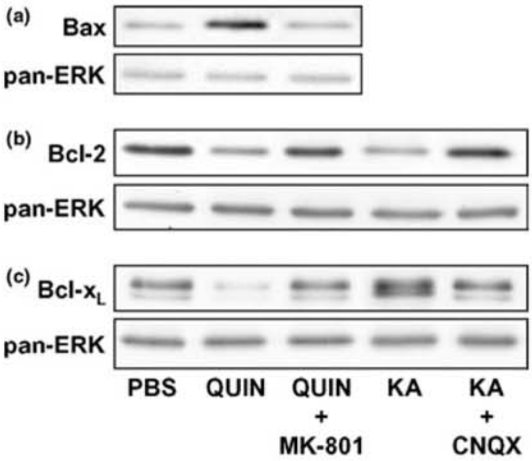
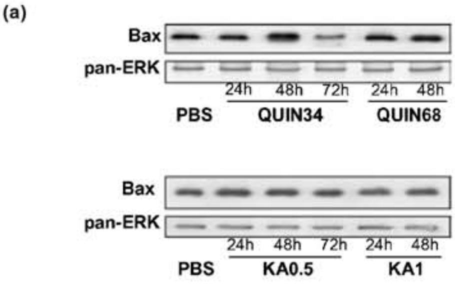


Fig. 4 Intrastriatal injection of glutamate receptor antagonists prevents changes in Bcl-2 family members induced by quinolinate (QUIN) or kainate (KA). The expression levels of Bax (a), Bcl-2 (b) and Bcl-x_L (c) proteins were examined by western blot after intrastriatal injection of QUIN (34 nmol), QUIN plus dizocilpine maleate (MK-801, 0.5 nmol), KA (1 nmol) or KA plus 6-cyano-7-nitroquinoxaline-2, 3-dione disodium (CNQX, 15 nmol). Bax and Bcl-2 were examined 48 h after injection, whereas Bcl-x_L was analyzed 48 h after KA and 72 h after QUIN injection. In each case the immunoblots shown are representative of four experiments.

stimulation. Only KA injection induced an increase in Bcl-x_L levels, which occurred earlier (24 h) in response to higher doses (Figs 3b and c). However, both glutamate receptor agonists down-regulated Bcl-x_L protein levels, the timing of the response being dependent upon the dose injected: at low doses the reduction was observed at 72 h whereas at high doses these changes were detected at 48 h (Figs 3b and c). Bcl-2 or Bcl-x_L protein levels were not modified by phosphate-buffered saline injection compared with non-injected striata (data not shown). All these effects could be blocked by specific glutamate receptor antagonists (Fig. 4). Hence, simultaneous injection of MK-801 with QUIN (34 nmol) prevented the decrease in Bcl-2 (Fig. 4b) and Bcl-x_L (Fig. 4c) protein levels induced by QUIN alone. Similarly, intrastriatal coinjection of CNQX with KA (0.5 nmol) inhibited the reduction in Bcl-2 (Fig. 4b) and the up-regulation of Bcl-x_L (Fig. 4c) protein levels induced by KA injection alone.

Intrastriatal injection of quinolinate but not kainate increases Bax levels

As observed for Bcl-2 and Bcl-x_L, phosphate-buffered saline injection did not modify Bax levels compared with the non-injected striata (data not shown). Bax protein levels were selectively regulated by QUIN and KA intrastriatal injection, with only QUIN injection inducing changes. Bax protein levels increased by 63% and 50%, 48 h after injection of low and high doses of QUIN, respectively (Fig. 5). This increase in Bax protein levels after QUIN was blocked by the glutamate receptor antagonist MK-801 (Fig. 4a).



(b)

	Bax		
	24 h	48h	72h
QUIN 34 nmol	92±1.2	163±2**	49±3**
QUIN 68 nmol	78±15	150±12*	N.D.
KA 0.5 nmol	80±11	112±6	78±17
KA 1 nmol	79±5	132±20	N.D.

Fig. 5 Intrastriatal injection of quinolinate (QUIN) but not kainate (KA) induces an increase in Bax protein levels. (a) Bax protein levels were analyzed by western blot at various times after intrastriatal injection of different doses of QUIN (34 nmol, QUIN34; 68 nmol, QUIN68) or KA (0.5 nmol, KA0.5; 1 nmol, KA1). Immunoblots show representative experiments. (b) Results obtained from densitometric measures are expressed as percentages of control (phosphate-buffered saline-injected striata) ± SEM for four animals per condition. $^{**}p < 0.01$; $^{*}p < 0.05$, compared with control values. Statistical analysis performed by one-way analysis of variance (ANOVA) followed by the Scheffé *post hoc* test. N.D., not determined

Differential reduction of quinolinate- and kainate-induced cell death in Bax null mutant mice

Our results showing that Bax protein levels were only modified by QUIN injection indicated that this protein could be differentially involved in the apoptotic cell death induced by NMDA or non-NMDA receptor stimulation. In order to study this possibility, cell death induced by QUIN or KA intrastriatal injection was examined in wild-type and Bax null mutant mice. Fluoro-Jade staining was used to identify degenerating neurons 48 h after injection. A specific signal corresponding to degenerating cells were observed in sections obtained from QUIN or KA injected animals (Fig. 6). Staining was characterized by virtually no background labeling of neuropil or unlesioned cells (Figs 6a and b), whereas degenerating neurons and their processes were highly stained (insets in Figs 6c–f). Analysis of the striatal volume occupied by Fluoro-Jade-positive cells disclosed that in wild-type mice QUIN and KA induced lesions

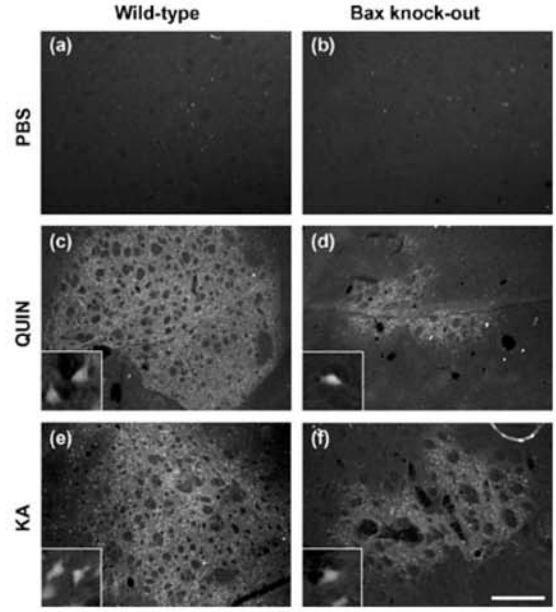


Fig. 6 Lesion size induced by excitotoxicity is reduced in Bax null mutant animals. Fluoro-Jade staining was performed 48 h after intrastriatal injection of phosphate-buffered saline (PBS), quinolinate (QUIN, 12 nmol) or kainate (KA, 0.5 nmol) in wild-type or Bax null mutant animals. Photomicrographs show striatal area occupied by Fluoro-Jade-positive neurons in wild-type (a, c and e) and Bax knock-out (b, d and f) animals injected with phosphate-buffered saline (a and b), QUIN (c and d) or KA (e and f). Insets in (c–f) show high-power images of Fluoro-Jade stained dying neurons. Scale bar indicates 280 μm for the lower magnification images and 40 μm for the insets.

of similar volume (1.61 ± 0.15 mm^3 and 1.95 ± 0.28 mm^3, respectively; Figs 6c and e), which were decreased in Bax null mutant mice (Figs 6d and f). However, the reduction was more pronounced in QUIN-injected (by 70 ± 8%; Fig. 6d) than in KA-injected (by 43 ± 5%; Fig. 6f) striata.

Quinolinate and kainate intrastriatal injection differentially modify Bax:Bcl-2 and Bax:Bcl-x_L heterodimerization

Although Bax protein levels were only modified by intrastriatal QUIN injection, results obtained in Bax null mutant animals indicate that the presence of Bax also participates in KA-induced apoptotic cell death. As the pro-apoptotic properties of Bax could also be regulated through heterodimerization with Bcl-2 and/or Bcl-x_L, we assessed the capacity of these proteins to heterodimerize with Bax in lesioned striata. This analysis was performed 48 h after QUIN injection, as Bax protein levels were increased at this time point, whereas in striata injected with KA, coimmunoprecipitation of Bcl-2 or Bcl-x_L with Bax was examined at 24 and 48 h after injection, because Bax protein levels were not modified by this glutamate receptor agonist. We chose high doses of QUIN (68 nmol) and KA (1 nmol) for these experiments because under these conditions Bcl-2 and Bcl-x_L were decreased at the same time point (48 h). Intrastriatal injection of QUIN and KA both reduced Bax:Bcl-2 heterodimerization (Fig. 7a). In contrast, intrastriatal injection of KA reduced the amount of Bcl-x_L that coimmunoprecipitated with Bax, whereas QUIN injection did not affect the levels of these heterodimers (Fig. 7b).

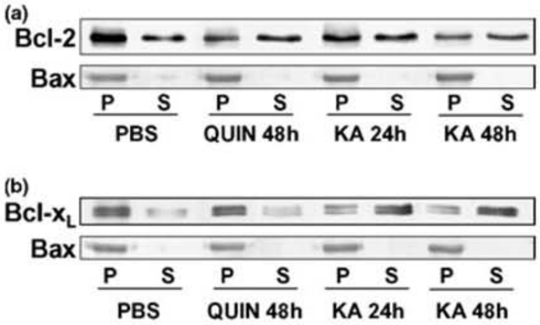


Fig. 7 Glutamate receptor agonists differentially modify Bax:Bcl-2 and Bax:Bcl-x_L heterodimer levels. Bax protein was immunoprecipitated with a polyclonal antibody against Bax and membranes were then subjected to immunoblotting with Bcl-2, Bcl-x_L or Bax antibodies. Proteins were obtained from striata injected with phosphate-buffered saline (PBS), quinolinate (QUIN, 68 nmol) or kainate (KA, 1 nmol). (a) Bax:Bcl-2 and (b) Bax:Bcl-x_L heterodimers at different time points after intrastriatal injection of phosphate-buffered saline, QUIN or KA. Immunoblots were obtained from representative experiments. P, pellet; S, supernatant.

Brain-derived neurotrophic factor prevents the decrease in Bcl-2 and Bcl-x_L protein levels induced by quinolinate and kainate

BDNF-secreting or control cells were grafted in the striatum to examine whether this neurotrophin protects striatal neurons against glutamate receptor agonist-induced damage via regulation of Bcl-2 family members. In striata grafted with BDNF-secreting cells prior to phosphate-buffered saline injection, we observed no difference from controls (F3A-MT plus phosphate-buffered saline-injected; Figs 8a and b). Intrastriatal injection of QUIN or KA in striata grafted with the control cell line decreased the levels of Bcl-2 (Fig. 8a) and Bcl-x_L (Fig. 8b). In contrast, when the BDNF-secreting cell line was grafted before QUIN or KA injection the decrease in protein levels was prevented. The reduction in Bcl-2 protein levels (Fig. 8a) was similarly prevented by BDNF in both KA and QUIN treatments. The prevention of the decrease in Bcl-x_L protein levels by BDNF, however, was stronger in striata injected with KA (by 95%; Fig. 8b) than in QUIN-injected striata (by 49%; Fig. 8b).

Bcl-2 and Bcl-x_L were also examined by immunohistochemistry at the same time points. Positive cells were

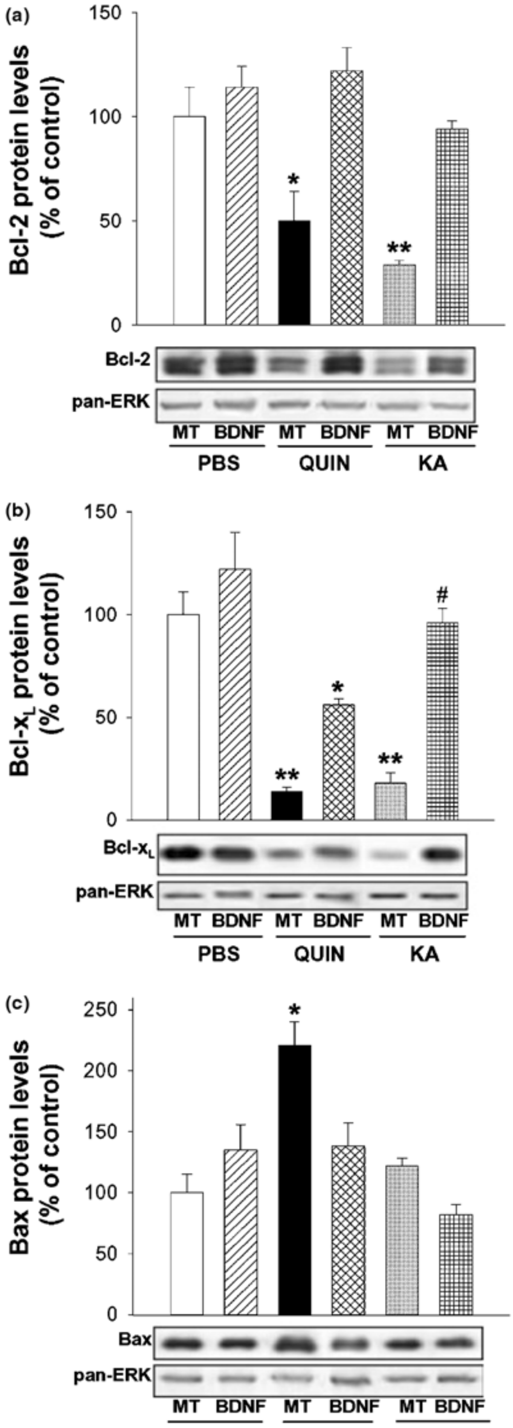


Fig. 8 Brain-derived neurotrophic factor (BDNF) prevents changes in Bcl-2, Bcl-x_L and Bax protein levels induced by excitotoxicity. Control (MT) or BDNF-secreting (BDNF) cell lines were intrastriatally grafted 24 h before injection of phosphate-buffered saline (PBS), quinolinate (QUIN, 34 nmol) or kainate (KA, 0.5 nmol). Bcl-2 (a) and Bax (c) protein contents were examined by western blot 48 h after injection, and Bcl-x_L at 72 h (b). Figures show the quantification of the blots with results expressed as percentages of control (striata grafted with control cell line plus PBS injection) ± SEM of four animals per condition. $^*p < 0.05$; $^{**}p < 0.01$, compared with control values; $\#p < 0.05$, compared with striata grafted with BDNF-secreting cells and injected with QUIN. Statistical analysis performed by one-way analysis of variance (ANOVA) followed by the Scheffé *post hoc* test. Immunoblots are shown for representative experiments.

analyzed in the penumbra zone, as Nissl staining reveals the presence of striatal neurons in this region after intrastriatal injection of QUIN (compare Figs 9a and b). Phosphate-buffered saline injection in striata grafted with the control cell line did not induce changes in Bcl-2 or Bcl-x_L immunoreactivity compared with non-injected striata (data not shown). In this experimental condition, weak Bcl-2 immunoreactivity was present in medium-sized projection neurons and in large interneurons (Fig. 9d). Intrastriatal injection of QUIN (Fig. 9e) or KA (data not shown) in striata grafted with the control cell line reduced the number of Bcl-2-positive neurons. In these animals, the number of Bcl-2-positive neurons in a region close to the injection site was 456 ± 34 cells/mm^2 (Fig. 9e), whereas the number of Nissl-stained neurons in the same region was 958 ± 103 cells/mm^2 (Fig. 9b). When compared with control animals, QUIN injection reduced the number Bcl-2-positive neurons by 78%, whereas the number of Nissl-stained neurons was only decreased by 53% (compare Fig. 9b with Fig. 9e). Thus, in QUIN injected striata about 25% of the neurons can be visualized by Nissl staining but not by immunohistochemistry against Bcl-2, suggesting a reduction in Bcl-2 protein levels per cell. As expected, grafting of BDNF-secreting cells prevented the reduction in Bcl-2-positive neurons induced by QUIN (Fig. 9f) and KA (data not shown) injection. Bcl-x_L immunoreactivity in control animals was only detected in large striatal interneurons (Fig. 9g) where it was localized in the cytoplasm. Intrastriatal injection of QUIN (Fig. 9h) or KA (data not shown) in striata grafted with the control cell line strongly decreased the number of positive cells (compare Figs 9h and b). Intrastriatal grafting of the BDNF-secreting cell line prevented the decrease in the number of Bcl-x_L-positive cells induced by QUIN (Fig. 9i) and KA (data not shown) intrastriatal injection. Interestingly, in striata grafted with the BDNF cell line and injected with QUIN (Fig. 9i) or KA (data not shown) Bcl-x_L was also expressed by medium-sized striatal neurons that survived the lesion.

Brain-derived neurotrophic factor blocks the increase in Bax protein levels induced by quinolinate

Bax protein levels were not modified by intrastriatal grafting of BDNF-secreting cells (Fig. 8c). As observed in non-grafted animals, QUIN injection in striata grafted with the

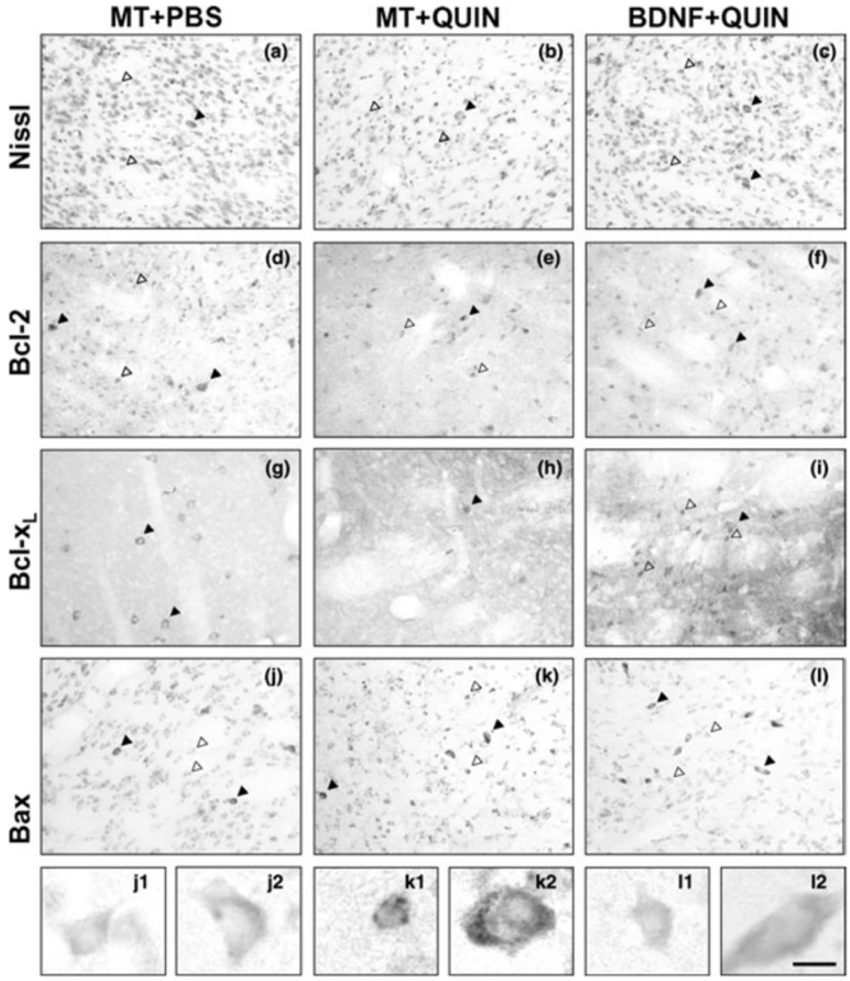


Fig. 9 Effects of quinolinate (QUIN) and brain-derived neurotrophic factor (BDNF) on striatal Bcl-2-, Bcl-x_L- and Bax-positive neurons. Bcl-2, Bcl-x_L and Bax proteins were examined by immunohistochemistry after intrastriatal injection of phosphate-buffered saline (PBS) or QUIN (34 nmol) in animals grafted with control (MT) or BDNF-secreting (BDNF) cells. Immunohistochemistry for Bcl-2 and Bax was performed 48 h after QUIN-induced excitotoxicity and for Bcl-x_L at 72 h. All photomicrographs show the penumbra zone. Nissl-stained sections revealed a slight decrease in the number of striatal cells in response to intrastriatal QUIN injection (b) compared with control (a), which was prevented by grafting of BDNF-secreting cells (c). In the control striatum (grafted with the control cell line and phosphate-buffered saline-injected), Bcl-2 (d) and Bax immunoreactivity (j) were observed in medium-sized (open arrowheads and j1) and in large (closed arrowheads and j2) striatal neurons, whereas only large striatal neurons showed Bcl-x_L immunoreactivity (g, closed arrowheads). Note the decrease in Bcl-2 (e) and Bcl-x_L (h) immunoreactivity induced by intrastriatal QUIN injection in striata grafted with the control cell line. Bax signaling was increased (k) and clustered (k1 and k2) in striata grafted with the control cell line and injected with QUIN. Grafting of BDNF-secreting cells blocked changes in Bcl-2 (f), Bcl-x_L (i) and Bax (l, l1 and l2) immunoreactivity induced by intrastriatal QUIN injection. Scale bar indicates 10 μm for panels showing high power magnification and 90 μm for panels (a–l).

control cell line increased Bax protein levels, whereas KA did not induce any change (Fig. 8c). In contrast, grafting of the BDNF-secreting cell line prevented the increase in Bax protein levels induced by QUIN (Fig. 8c).

Immunohistochemical analysis of control animals (non-lesioned or grafted with the control cell line prior to phosphate-buffered saline injection) showed that Bax protein was localized in the cytoplasm of medium-sized (Fig. 9j1) and large striatal neurons (Fig. 9j2) showing diffuse immunostaining. Forty-eight hours after intrastriatal injection of QUIN in animals grafted with the control cell line, Bax immunoreactivity was increased in both medium-sized and large striatal neurons (Fig. 9k) and displayed a punctate pattern (Figs 9k1 and k2). Changes in the immunostaining pattern were more evident in medium-sized striatal neurons, in which Bax immunoreactivity was concentrated in small clusters (Fig. 9k1). Intrastriatal grafting of the BDNF cell line counteracted changes in Bax immunoreactivity induced by QUIN. Under these conditions, Bax immunolabeling was similar to that of controls (Figs 9l, l1 and l2).

Brain-derived neurotrophic factor prevents quinolinate- and kainate-induced changes in Bax:Bcl-2 and Bax:Bcl-x_L heterodimerization

As intrastriatal injection of QUIN or KA caused changes in Bax:Bcl-2 and Bax:Bcl-x_L heterodimerization, we analyzed whether BDNF could prevent these changes. Intrastriatal grafting of the control cell line did not modify the effect of QUIN or KA on the heterodimerization of Bcl-2 proteins (data not shown). As observed with higher doses, intrastriatal injection of QUIN (Fig. 10a) or KA (Fig. 10b) in striata grafted with the control cell line reduced Bax:Bcl-2 heterodimerization. KA injection decreased the coimmunoprecipitation of Bcl-2 and Bax as early as 24 h after injection, but was particularly apparent at 48 h (Fig. 10b). Intrastriatal grafting of BDNF-secreting cells before QUIN or KA injection increased the levels of Bcl-2 that coimmunoprecipitated with Bax (Figs 10a and b). Bax:Bcl-x_L heterodimerization was not affected by QUIN injection in striata grafted with control or BDNF-secreting cells (Fig. 10a). In contrast, KA injection in striata grafted with the control cell line reduced the amount of Bcl-x_L that coimmunoprecipitated with Bax, Bcl-x_L being increased in the supernatants (Fig. 10b). As observed for Bax:Bcl-2 heterodimerization, the decrease was stronger at 48 h than at 24 h after KA injection (Fig. 10b). The reduction in the levels of Bax:Bcl-x_L heterodimerization induced by KA was prevented by intrastriatal grafting of the BDNF-secreting cell line (Fig. 10b).

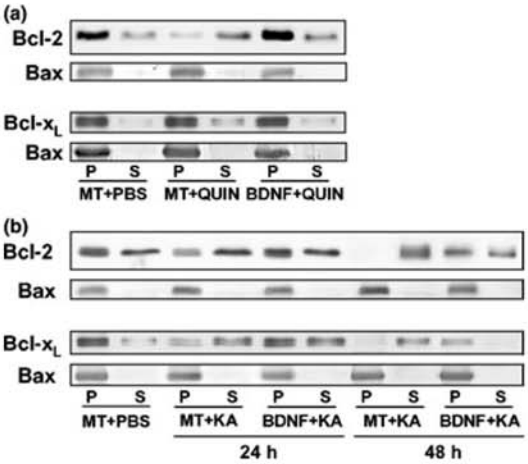


Fig. 10 Brain-derived neurotrophic factor (BDNF) effects on changes in Bax:Bcl-2 and Bax:Bcl-x_L heterodimers induced by quinolinate (QUIN) or kainate (KA) intrastriatal injection. Heterodimer levels were examined by immunoprecipitation in striata grafted with control (MT) or BDNF-secreting (BDNF) cell lines and injected with phosphate-buffered saline (PBS), QUIN (34 nmol) or KA (0.5 nmol). (a) Bax:Bcl-2 and Bax:Bcl-x_L heterodimers 48 h after intrastriatal injection of PBS or QUIN. (b) Bax:Bcl-2 and Bax:Bcl-x_L heterodimers 24 and 48 h after intrastriatal injection of PBS or KA. Immunoblots were obtained from representative experiments. P, pellet; S, supernatant.

Brain-derived neurotrophic factor blocks quinolinate- and kainate-induced caspase-3 activation

We next determined whether the intrastriatal injection of QUIN or KA affects caspase-3 activity and whether BDNF regulates such activation. Our results showed that Ac-DEVD-acf cleavage was enhanced (30%) by QUIN or KA intrastriatal injection, but at different time points (Fig. 11a). Caspase-3 activity rose 48 h after QUIN injection, whereas after KA injection the increase was first

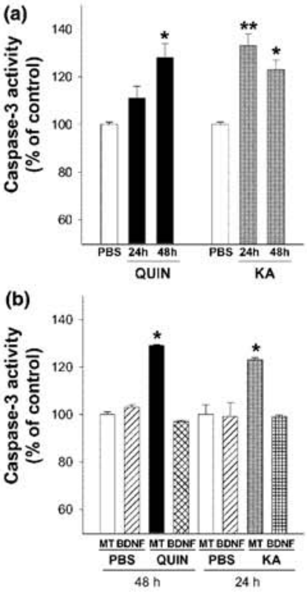


Fig. 11 Caspase-3 activity induced by quinolinate (QUIN) and kainate (KA) is reduced in the presence of brain-derived neurotrophic factor (BDNF). DEVD cleavage assay was performed to determine caspase-3 activity. (a) Bars showing Ac-DEVD-acf cleavage in striata at 24 and 48 h after intrastriatal injection of phosphate-buffered saline (PBS), QUIN (34 nmol) or KA (0.5 nmol). (b) Bars showing caspase-3 activity examined in animals grafted with control (MT) or BDNF-secreting (BDNF) cell lines and injected with PBS, QUIN or KA. The DEVD assay was performed 24 h after PBS or KA injection and 48 h after PBS or QUIN injection. Results expressed as percentages ± SEM of control values (PBS or MT + PBS injected striata) were obtained from three animals per condition. $^*p < 0.05$; $^{**}p < 0.01$ compared with control values. Statistical analysis performed by one-way analysis of variance (ANOVA) followed by the Scheffé *post hoc* test.

detected at 24 h and maintained at 48 h, the last time-point examined. In grafted animals the analysis of caspase-3 activation was performed 24 h after phosphate-buffered saline or KA and 48 h after phosphate-buffered saline or QUIN intrastriatal injection. Intrastriatal grafting of both cell lines did not modify caspase-3 activity in animals injected with phosphate-buffered saline (Fig. 11b). Similarly, intrastriatal grafting of the control cell line did not alter the Ac-DEVD-acf cleavage induced by QUIN or KA injection (compare Figs 11a and b). In contrast, caspase-3 activation was prevented when BDNF-secreting cells were grafted in the striatum 24 h before QUIN or KA injection (Fig. 11b).

Discussion

The present study was designed to test the hypothesis that the neuroprotective properties of BDNF against excitotoxicity in striatal neurons are mediated by regulation of members of the Bcl-2 protein family. Stimulation of NMDA or non-NMDA receptors in the striatum results in a similar morphological pattern of cell death and decrease in p-Akt protein levels. However, Bcl-2 family protein levels and heterodimerization are differentially regulated. Intrastriatal injection of QUIN, but not KA, increases Bax pro-apoptotic protein levels, whereas only KA injection down-regulates Bax:Bcl-x_L heterodimer levels. Furthermore, using Bax null mutant mice we have shown a differential involvement of this pro-apoptotic protein in QUIN- and KA-induced apoptotic cell death. Although Bcl-2 family proteins are differentially regulated by NMDA and non-NMDA receptor stimulation, these changes ultimately lead to caspase-3 activation that may account for the morphological similarity of cell death observed after the intrastriatal injection of QUIN or KA. Intrastriatal grafting of a BDNF-secreting cell line prevents changes in the p-Akt, Bcl-2 family members and caspase-3 activation induced by NMDA and non-NMDA receptor activation.

Our results show that intrastriatal injection of QUIN or KA similarly induced the appearance of apoptotic profiles. In agreement with our results, it has been shown previously that intrastriatal injection of different doses of QUIN (Hughes *et al*. 1996; Bordelon *et al*. 1999; Wei *et al*. 2002) and KA (Ferrer *et al*. 1995; Lok and Martin 2002) cause apoptotic cell death. Under these experimental conditions, BDNF is neuroprotective for striatal neurons (Perez-Navarro *et al.* 1999, 2000a; Gratacos *et al.* 2001). Furthermore, BDNF exerts its trophic effects on cultured striatal neurons through the activation of the PI3K/Akt pathway (Gavalda *et al.* 2004). Our results confirm the involvement of this intracellular signaling pathway in the BDNF neuroprotective effects against excitotoxicity showing that stimulation of NMDA and non-NMDA receptors induced a transient loss of p-Akt protein that was prevented by BDNF treatment. Similarly, previous studies have shown reduced p-Akt levels in response to glutamate receptors stimulation (Chalecka-Franaszek and Chuang 1999; Luo *et al.* 2003) and several protective agents prevent cell death by activating the PI3K/Akt pathway (Sawada *et al.* 2000; Yamagishi *et al.* 2003). These results suggest that decreased Akt activation has consequences for downstream targets, such as Bcl-2 family proteins, involved in cell survival (Skorski *et al.* 1997; Riccio *et al.* 1999).

To test the contribution of Bcl-2 family members to excitotoxicity-induced neuronal death, their levels were analyzed following intrastriatal injection of QUIN or KA. A distinct short-term up-regulation of Bcl-2 and Bcl-x_L was observed after QUIN and KA intrastriatal injection. Bcl-x_L protein levels were increased only by the stimulation of non-NMDA receptors, whereas Bcl-2 levels were enhanced by both glutamate receptor agonists. The increase in these anti-apoptotic protein levels may be interpreted as an endogenous neuroprotective response of striatal neurons to excitotoxicity. In agreement with this, it has shown previously that excitotoxic lesion of the striatum induces a transient up-regulation of the expression of several neurotrophic factors and their receptors (Canals *et al.* 1998, 1999; Marco *et al.* 2002). However, this short-term increase in survival proteins is insufficient to prevent striatal neuronal death. In fact, Bcl-2 and Bcl-x_L protein levels were down-regulated at later times after both QUIN and KA injection suggesting that apoptotic cell death may, in part, be related to reduced levels of anti-apoptotic proteins. Similarly, previous studies have shown decreased Bcl-2 and/or Bcl-x_L mRNA or protein levels after various types of injuries that lead to apoptotic cell death (Krajewski *et al.* 1995; Sato *et al.* 1998; Tamatani *et al.* 1998; Ghribi *et al.* 2002; Wei *et al.* 2002). It is also noteworthy that immunohistochemical analysis of these anti-apoptotic proteins revealed that in control animals all striatal neurons express Bcl-2, but Bcl-x_L is only present in large striatal interneurons. This cell specificity may explain the differential vulnerability of striatal neuronal populations to excitotoxicity (Davies and Roberts 1988; DiFiglia 1990; Perez-Navarro *et al.* 2000b; Gratacos *et al.* 2001).

In this study we found that the presence of Bax is differentially required in the apoptotic cell death induced by NMDA and non-NMDA receptor stimulation in the striatum. Only QUIN injection increased Bax protein levels. Furthermore, in Bax null mutant animals, the volume of the lesion induced by QUIN and KA was decreased, being the reduction more evident in animals injected with QUIN. In addition to the regulation of protein levels, Bax function can also be modified by heterodimerization with Bcl-2 and Bcl-x_L (Cory and Adams 2002). Both QUIN and KA intrastriatal injection reduced the levels of Bax:Bcl-2 heterodimers, whereas Bax:Bcl-x_L heterodimer levels were only modified by KA. It is interesting to note that reduced Bax:Bcl-x_L and Bax:Bcl-2 heterodimer levels 24 h after KA injection were not accompanied by changes in protein levels, suggesting that the relative levels of Bcl-2 family proteins are less likely to contribute to the induction of neuronal death following non-NMDA receptor stimulation. In contrast, the decrease in Bax:Bcl-2 heterodimer levels after intrastriatal injection of QUIN is accompanied by changes in Bax and Bcl-2 protein levels. Similarly, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dopaminergic neuron damage is mediated by an increase in Bax and a decrease in Bcl-2 protein levels that reduces Bax:Bcl-2 heterodimerization (Vila *et al*. 2001). In contrast to our results, a previous study shows that transgenic mice overexpressing Bcl-2 do not exhibit reduced sensitivity to QUIN-induced striatal cell death (Maciel *et al*. 2003). This contradictory result could be related to the dose of QUIN injected, because the dose of QUIN used in that study (Maciel *et al*. 2003) is fourfold the dose of QUIN injected in the present work. It is known that excitotoxicity can induce both necrotic and apoptotic cell death depending on the intensity and duration of the insult (Ankarcrona *et al*. 1995; Bonfoco *et al*. 1995; Ayata *et al*. 1997). Thus, high doses of QUIN are prone to kill cells by a necrotic mechanism not involving Bcl-2 family members.

Taken together, our data suggest that activation of Akt and Bcl-2 family expression pathway is responsible, at least in part, for the BDNF-protective effect on striatal neurons against excitotoxicity. In agreement with our results, it has been reported that the neuroprotective effect of BDNF after focal cerebral ischemia is mediated by reduced Bax expression and up-regulation of Bcl-2 levels (Schabitz *et al*. 2000), and that the enhancement (Allsopp *et al*. 1993) or inhibition (Allsopp *et al*. 1995) of endogenous Bcl-2 expression regulates the survival response of the trigeminal mesencephalic neurons to BDNF. However, our results also show that KA-induced cell death is mediated by changes in the interaction between Bax and both Bcl-2 and Bcl-x_L, without changes in their expression levels. As the anti-apoptotic function of Bcl-2 and Bcl-x_L can be inactivated by phosphorylation (Haldar *et al*. 1996; Fan *et al*. 2000) we can not rule out the involvement of other intracellular signaling pathways that can be activated by excitotoxicity. In this regard, glutamate receptor stimulation activates c-Jun N-terminal kinase (Schwarzschild *et al*. 1997; Yang *et al*. 1997; Mielke *et al*. 1999), a kinase that has been shown to phosphorylate Bcl-2 and Bcl-x_L (Maundrell *et al*. 1997; Fan *et al*. 2000). Furthermore, Akt kinase activity can antagonize c-Jun N-terminal kinase activity (Okubo *et al*. 1998; Sarmiere and Freeman 2001; Kim *et al*. 2002). Therefore, it is tempting to speculate that BDNF can prevent excitotoxic cell death in the striatum by regulating the Bcl-2 family proteins through both transcription-dependent and independent mechanisms, as a result of the glutamate receptor type stimulated, thereby preventing Bax translocation to the mitochondria and subsequent caspase-3 activation.

In conclusion, our results show that NMDA and non-NMDA receptor stimulation in the striatum reduces p-Akt levels and selectively regulates Bax, Bcl-2 and Bcl-x_L protein levels and heterodimerization that lead to caspase-3 activation. BDNF blocks the decrease in p-Akt suggesting that modulation of PI3K/Akt pathway may provide a means to prevent changes in the Bcl-2 family members and subsequent caspase-3 activation induced by excitotoxicity.

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