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BDNF regulates BIM expression levels in 3-nitropropionic acid-treated cortical neurons

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ABSTRACT

3-Nitropropionic acid (3-NP) is an irreversible inhibitor of succinate dehydrogenase that has been used to explore the primary mechanisms of cell death associated with mitochondrial dysfunction and neurodegeneration in Huntington's disease. In this study we investigated the ability of brain-derived neurotrophic factor (BDNF) to suppress mitochondrial-dependent cell death induced by 3-NP in primary cortical neurons. This neurotrophin prevented 3-NP-induced release of cytochrome *c* and Smac/Diablo, caspase-3-like activity and nuclear condensation/fragmentation. Furthermore, it greatly increased phosphorylation of Akt and MAPK, suggesting the involvement of these signalling pathways in BDNF neuroprotection. Interestingly, BDNF decreased the levels of the pro-apoptotic protein Bim in mitochondrial and total cell lysates through the activation of the MEK1/2 pathway. This effect was due to an increase in the degradation rates of Bim. Our data support an important role for BDNF, in protecting cortical neurons against apoptotic cell death caused by inhibition of mitochondrial complex II.

Introduction

Mitochondrial dysfunction has been linked to the pathogenic mechanisms of several neurodegenerative diseases (Schapira, 2006; Keating, 2008). Neurons are particularly sensitive to alterations in normal mitochondrial function because of their high levels of activity and subsequent need for energy. 3-Nitropropionic acid (3-NP), an inhibitor of succinate dehydrogenase (Coles et al., 1979; Huang et al., 2006), has been widely used to analyse the mechanisms by which metabolic impairment leads to the degeneration of neurons. In particular, 3-NP has been used in the context of Huntington's disease (HD), a hereditary neurodegenerative disease affecting the striatum and the cerebral cortex, since its administration in rodents and non-human primates causes abnormal movement, cognitive deficits and neuronal degeneration similar to that seen in HD patients (Beal et al., 1993; Brouillet et al., 1995; Blum et al., 2002). In addition, post-mortem brain extracts of HD patients showed reduced activity and expression levels of succinate dehydrogenase (Gu et al., 1996; Browne et al., 1997; Benchoua et al., 2006). Although not entirely elucidated, the mechanisms of 3-NP-induced neurotoxicity involve depletion of ATP, mitochondrial membrane depolarization, dysregulation of intracellular calcium homeostasis, calpain activation, and release of pro-apoptotic proteins from mitochondria with the consequent activation of caspases and apoptotic pathways (Lee et al., 2002; Bizat et al., 2003; Almeida et al., 2004, 2006). Although these changes may underlie massive striatal degeneration following 3-NP *in vivo* administration, a reduction in the activities of complexes I and II was also recently described in the cerebral cortex (Pandey et al., 2008), implicating neuronal dysfunction in this brain area. Indeed, early cortical dysfunction, linked to striatal excitotoxicity and decreased trophic support, appears to have a fundamental role in the onset and progression of HD.

The mitochondrial death pathway is regulated by a fine balance between pro-apoptotic and pro-survival Bcl-2 family members (Cory and Adams, 2002). Pro-apoptotic proteins such as Bax and Bak can disrupt the outer mitochondrial membrane and promote the release of apoptogenic factors such as cytochrome *c* or Smac/Diablo, an event that ultimately activates the caspase cascade. Apoptosis-inducing factor (AIF) can also be released from the mitochondria, leading to caspase-independent cell death (Susin et al., 1999). In viable cells and under normal conditions, pro-apoptotic proteins are normally repressed by binding to pro-survival proteins such as Bcl-2 and Bcl-xL. In response to stress, BH3-only proteins, such as Bim, Bid, and Bad, bind to Bcl-2 or Bcl-xL, that then release Bax or Bak. These pro-apoptotic proteins then change their conformation and oligomerize in the mitochondrial membrane to promote cell death (Puthalakath and Strasser, 2002).

Trophic support to neurons largely influences neuronal survival and function. Members of the neurotrophin family, namely brain-derived neurotrophic factor (BDNF), have been suggested as therapeutic candidates to treat neurodegenerative disorders because they promote neuronal survival in different lesion models (e.g. Connor and Dragunow, 1998). BDNF is particularly relevant in HD since its transcription (Zuccato et al., 2001) and axonal transport (Gauthier et al.,

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2004) are decreased by the presence of mutant huntingtin, affecting the survival of both striatal and cortical neurons. BDNF was previously shown to prevent the death of different populations of striatal projection neurons in a quinolinic acid model of HD (Perez-Navarro et al., 2000; Kells et al., 2004). BDNF was also reported to protect striatal neurons from 3-NP toxicity (Ryu et al., 2004). However, the mechanism used by BDNF leading to neuroprotection is not clear. The effects of BDNF are mainly mediated by TrkB receptor-induced activation of key signalling pathways, including PLC-γ, Ras/MEK/MAPK and PI3K/Akt pathways (Huang and Reichardt, 2001). These pathways have been shown to regulate apoptotic cell death by increasing the transcription of neuroprotective proteins such as Bcl-2 (Pugazhenthi et al., 2000) and/or by posttranslational modifications of proteins such as Bad and Bim (del Peso et al., 1997; Scheid et al., 1999; Luciano et al., 2003; Qi et al., 2006).

Taking into account the importance of cortical dysfunction in HD and the potential benefit of BDNF against mitochondrial-driven neuronal degeneration, in this study we analysed the mechanisms by which BDNF protects primary cortical neurons against mild neurotoxicity induced by 3-NP. Our data show that BDNF protects cortical neurons from 3-NP toxicity through the activation of PI3K and MEK1/2 intracellular signalling pathways and the regulation of Bim turnover.

Materials and methods

Materials

Neurobasal medium and B-27 supplement were purchased from GIBCO (Paisley, UK). 3-Nitropropionic acid and anti-α-tubulin were from Sigma Chemical Co. (St. Louis, MO, USA). N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) was obtained from Calbiochem (Darmstadt, Germany). BDNF, Wortmannin and LY294002 were from Alomone Labs (Jerusalem, Israel). PD980859, K252a and K252b were from Calbiochem (Darmstadt, Germany). Hoechst 33342 was from Molecular Probes (Eugene, OR, USA). All other reagents were of analytical grade.

Cell culture

Primary neuronal cultures of rat cerebral cortical neurons were prepared as previously described (Almeida et al., 2004). In brief, cerebral cortices were dissected from fetal rats of the Wistar strain at 16–17 days-old and the cells dissociated. Cortical neurons were resuspended in Neurobasal medium supplemented with 2% B-27, 0.2 mM glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin and cultured in a humidified incubator at 37 °C and 5% CO_2. After five to seven days in culture, neurons were pre-treated with BDNF (50 or 100 ng/mL) for 30 min and further incubated with 0.3 mM 3-NP for 24 h (3-NP stock solution was prepared in water and brought to pH 7.4 with NaOH). Both compounds remained in the medium for the entire duration of the experiment. Where indicated, LY294002 (5 μM) and Wortmannin (50 nM), two PI3K inhibitors, PD980859 (30 μM) an inhibitor of MEK1/2, K252a (500 nM) and K252b (400 nM), two inhibitors of tyrosine kinase, and cycloheximide (10 μM) were incubated 1 h before the addition of BDNF and all compounds remained in the medium for the entire duration of the experiment.

Caspase protease activity assay

Caspase-like activity was determined as described previously (Almeida et al., 2004). Briefly, cortical cells were lysed and centrifuged at 14,000 rpm (Eppendorf Centrifuge 5417R) for 10 min. The resulting supernatants were collected and assayed for protein content using the Bio-Rad reagent, according to the manufacturer's instructions. Caspase-3-like activity was measured using Ac-DEVD-pNA as substrate. Caspase-like activity was calculated as the increase above the control, for equal protein loading (25 μg).

Nuclear morphology assay

After treatment, Neurobasal medium was replaced and cortical neurons remained in culture for an additional 24 h without BDNF or 3-NP. The cells were fixed with 4% paraformaldehyde (pH 7.4) and stained with Hoechst 33342 (7.5 μg/mL). The nuclear morphology of cortical neurons was analysed by fluorescence microscopy (Zeiss Axioskop 2 microscope). Fluorescence of stained chromatin was examined, and fragmented and/or condensed chromatin scored.

Subcellular fractionation

Total cell lysate

Cells were washed twice in ice-cold PBS and resuspended in lysis buffer (in mM: 20 Tris (pH 7.0), 100 NaCl, 2 EDTA, 2 EGTA, 1% Triton X-100) supplemented with 50 mM NaF, 1 mM Na_3VO_4, 1 mM 1,4-dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μg/mL protease inhibitor cocktail (chymostatin, pepstatin A, leupeptin and antipain). The lysates were sonicated on ice for 30 s and centrifuged (14,000 rpm for 10 min, Eppendorf Centrifuge 5417R) to remove insoluble material. The supernatants were collected and stored at −80 °C.

Mitochondrial and cytosolic fractions

Cells were washed twice in ice-cold PBS and resuspended in sucrose buffer (in mM: 250 sucrose, 20 HEPES/KOH (pH 7.5), 100 KCl, 1.5 $MgCl_2$, 1 EGTA and 1 EDTA) supplemented with 1 mM DTT, 0.1 mM PMSF and 1 μg/mL protease inhibitor cocktail on ice. Lysates were homogenized and centrifuged at 500 × *g* for 12 min (4 °C) to pellet the nuclei and cell debris. The supernatant was further centrifuged at 12,000 × *g* for 20 min. The resulting pellet (mitochondrial fraction) was resuspended in TNC buffer (10 mM Tris acetate, pH 8.0, 0.5% Nonidet P-40, 5 mM $CaCl_2$) containing protease inhibitors. TCA (15%) was added to the 12,000 × *g* supernatant and centrifuged at 15,800 × *g* for 10 min. The resulting pellet (cytosolic fraction) was resuspended in supplemented sucrose buffer and brought to pH 7 with KOH. Samples were frozen at −80 °C.

Analysis of mitochondrial adenine nucleotides

Mitochondrial fractions were prepared and assayed for adenine nucleotides (ATP, ADP and AMP) by separation in a reverse-phase high performance liquid chromatography (HPLC) as described previously (Rego et al., 1997).

Citrate synthase assay

Citrate synthase (CS) activity was performed at 412 nm following the reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) in the presence of acetyl-CoA and oxaloacetate, according to Coore et al. (1971). CS activity was expressed in nanomoles per minute per milligram of protein.

Mitochondrial respiratory chain complex activities

NADH–ubiquinone oxidoreductase assay

Complex I activity was determined at 340 nm by following the decrease in NADH absorbance that occurs when ubiquinone is reduced to ubiquinol, according to Ragan et al. (1987). Complex I activity was expressed in nanomoles per minute per milligram of protein and corresponds to the rotenone sensitive rate. The enzyme activity was normalised to citrate synthase activity.

Succinate–ubiquinone oxidoreductase assay

Complex II activity was monitored at 600 nm by following the reduction of 6,6-dichlorophenolindophenol (DCPIP) by the ubiquinol formed in the reaction, according to Hatefi and Stiggall (1978). Complex II activity was expressed in nanomoles per minute per milligram of protein and corresponds to the TTFA sensitive rate. The enzyme activity was normalised to citrate synthase activity.

Ubiquinol–cytochrome c reductase assay

Complex III activity was monitored at 550 nm by following the reduction of cytochrome *c* by ubiquinol. The assay was started by adding the sample to the reaction mixture (in mM: 35 K_2HPO_4, pH 7.2, 1 EDTA, 5 $MgCl_2$, 1 KCN, and 5 μM rotenone) containing 15 μM cytochrome *c* and 15 μM ubiquinol, at 30 °C. Complex III activity was expressed in rate constant (*k*) per minute per milligram of protein and normalised to citrate synthase activity.

Cytochrome c oxidase assay

Complex IV activity was determined at 550 nm by measuring the oxidation of reduced cytochrome *c* by cytochrome *c* oxidase, according to Wharton and Tzagotoff (1967). Complex IV activity was expressed in rate constant (*k*) per minute per milligram of protein and normalised to citrate synthase activity.

Western blotting analysis

Subcellular fractions were prepared from cortical neurons as described above and protein content was determined using the Bio-Rad reagent. Equivalent amounts of protein were separated on 10–15% SDS-PAGE gels and electroblotted onto polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Buckinghamshire, UK). After blocking with 5% skim milk in TBS (25 mM Tris/HCl, pH 7.6, 150 mM NaCl)/0.1% Tween, for 2 h (RT), the membranes were incubated with antibodies directed against the denatured form of cytochrome *c* (PharMingen, 1:500), Smac/Diablo (BD Bioscience, 1:500), Bcl-2 (Santa Cruz Biotechnology Inc., 1:500), AIF (Santa Cruz Biotechnology Inc., 1:1000), P-Akt (Cell Signaling, 1:1000), P-MAPK (Cell Signaling, 1:1000), Akt (BD Bioscience, 1:1000), MAPK (Cell Signaling, 1:1000), and Bim (Stressgen, 1:500) overnight, at 4 °C. The membranes were further incubated with the secondary antibody (1:20,000) for 2 h (RT) and the proteins were visualized by using an enhanced chemifluorescence reagent (Amersham Biosciences). The antibody directed against α-tubulin (1:30,000) was used to normalise the amount of protein per lane in total, cytosolic and mitochondrial fractions. Tubulin is an inherent component of mitochondrial membranes (Carré et al., 2002) and its levels did not change in any of the treatments used in this study.

Statistical analysis

Data were expressed as mean ± SEM of the number of experiments indicated in the figure legends. Comparisons among multiple groups were performed with one-way analysis of variance (ANOVA) followed by the Tukey–Kramer post-hoc test. Significance was defined as $p<0.05$.

Results

BDNF prevents mitochondrial-dependent apoptosis induced by 3-NP

In previous work we showed that 0.3 mM 3-NP activates the mitochondrial apoptotic pathway and that this concentration of 3-NP is not associated with loss of plasma membrane integrity and thus, massive cell death (Almeida et al., 2004). In the present study we determined whether BDNF protected cortical neurons against the apoptotic cell death induced by 3-NP. This concentration of 3-NP inhibited SDH activity by approximately 75% ($p<0.001$), but had no effect on mitochondrial ATP, ADP and AMP levels (Table 1). BDNF treatment alone or in combination with 3-NP did not affect SDH activity or adenine nucleotide levels (data not shown). Because the apoptotic process is dependent on the maintenance of the ATP levels in the cell, these results support the occurrence of apoptotic rather than necrotic cell death.

Based on previously reported neuroprotective effects of BDNF (e.g. Perez-Navarro et al., 2000), we analysed the effects of BDNF on caspase-3 activation induced by 0.3 mM 3-NP. As shown in Fig. 1A, BDNF (100 ng/mL) significantly reduced 3-NP-induced caspase-3 activation by approximately 25% ($p<0.05$). However, BDNF at a lower concentration (50 ng/mL) did not significantly protect neurons from 3-NP-induced toxicity (Fig. 1A). Although the neurotrophin alone seemed to decrease caspase-3 activation, the data was not statistically significant when compared to untreated neurons.

Because the active form of caspase-3 can lead to the activation of nuclear DNAses, which then cleave DNA, we tested the effects of BDNF (100 ng/mL) on 3-NP-induced chromatin condensation/fragmentation. In the presence of 3-NP, there was a decrease of approximately 20% ($p<0.001$) in the number of viable cells (showing no condensed and/or fragmented chromatin) relative to untreated neurons (Fig. 1B). Under these experimental conditions, BDNF prevented the decrease in viable cells induced by 3-NP (Fig. 1B).

Activation of PI3K and MEK1/2 signalling pathways upon treatment with BDNF

To specifically elucidate the mechanism by which BDNF protects neurons from 3-NP-induced cell death, we analysed the activation of the PI3K and MEK1/2 pathways by following the changes in the phosphorylation status of Akt and MAPK (ERK1/2), respectively. BDNF treatment alone significantly increased Akt phosphorylation by 78% ($p<0.01$, Fig. 2A). In the presence of 3-NP, BDNF increased Akt phosphorylation to levels greater than 220% ($p<0.05$) of untreated cells. This increase was not statistically significant when compared to BDNF alone. Under these experimental conditions, MAPK phosphorylation largely increased in BDNF-treated neurons (p44: 1181%, $p<0.001$ and p42: 293%, $p<0.05$, Fig. 2B). BDNF also elevated the phosphorylation of MAPK in cells treated with 3-NP (p44: 1291%, $p<0.05$ and p42: 262%). The incubation of neurons with LY294002 (a PI3K inhibitor) completely blocked Akt phosphorylation, but not the phosphorylation of MAPK in 3-NP plus BDNF-treated neurons. Interestingly, wortmannin, another PI3K

Table 1
Activity of mitochondrial respiratory chain complexes and adenine nucleotides levels.

	Control	3-NP
Complex activities		
Cx1/CS	7.774 ± 1.149	10.64 ± 2.383
Cx2/CS	43.11 ± 2.270	10.92 ± 0.268***
Cx3/CS	7.920 ± 1.173	7.160 ± 1.002
Cx4/CS	8.197 ± 0.375	7.360 ± 0.954
Citrate synthase (CS)	131.2 ± 14.71	130.2 ± 17.76
Adenine nucleotides		
ATP	22.70 ± 2.628	22.86 ± 4.139
ADP	14.70 ± 2.967	8.990 ± 2.527
AMP	8.020 ± 2.642	5.817 ± 1.649

Cortical neurons were incubated in the absence (control) or in the presence of 0.3 mM 3-NP, for 24 h. Mitochondrial respiratory chain complex (Cx1–4) activities were corrected for citrate synthase activity (CS). Values are given in nmol/min/mg protein, except for Cx3 and Cx4, which are given in *k*/min/mg protein. The adenine nucleotides were measured in mitochondrial fractions prepared as indicated in Materials and methods and the values are given in nmol/mg protein. The results were expressed as the mean ± SEM of 3 distinct experiments, performed in duplicate. Statistical analysis: ***$p<0.001$, compared to untreated neurons.

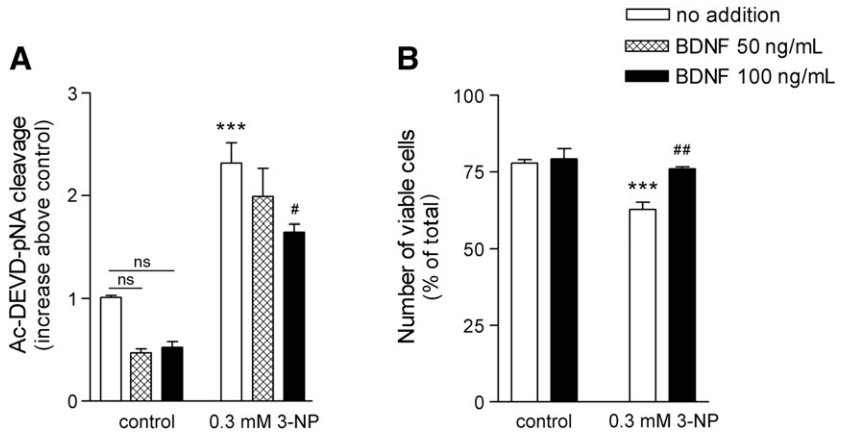

Fig. 1. Effects of BDNF on 3-NP-mediated cell death. Cortical neurons were pre-treated with BDNF (50 and 100 ng/mL) for 30 min and further incubated in the absence or presence of 0.3 mM 3-NP for 24 h. (A) Caspase-3-like activity was measured by following the cleavage of the colorimetric substrate Ac-DEVD-pNA. The activity is expressed as the increase of optical density values above the control (untreated neurons). (B) Cells were stained with Hoechst 33342 and assayed for nuclear morphology. Cell viability is expressed as a percentage of the total number of cells (approximately 100 cells per field were counted). Results were expressed as the mean ± SEM of 4–10 distinct experiments. Statistical analysis: ***$p<0.001$, compared to untreated neurons; $^{\#}p<0.05$, $^{\#\#}p<0.01$, compared to 3-NP-treated neurons in the absence of BDNF; ns, not significant.

inhibitor, only partially decreased Akt phosphorylation induced by BDNF in the presence of 3-NP and did not affect MAPK phosphorylation (Figs. 2A, B). The concentration of wortmannin used in this study was previously shown to completely block PI3K activity in cortical neurons (Mannella and Brinton, 2006). However, because wortmannin was previously reported to lose effectiveness after 5 h in serum-containing cultures (Kimura et al., 1994), it is possible that after 24 h incubation, the PI3K was not maximally

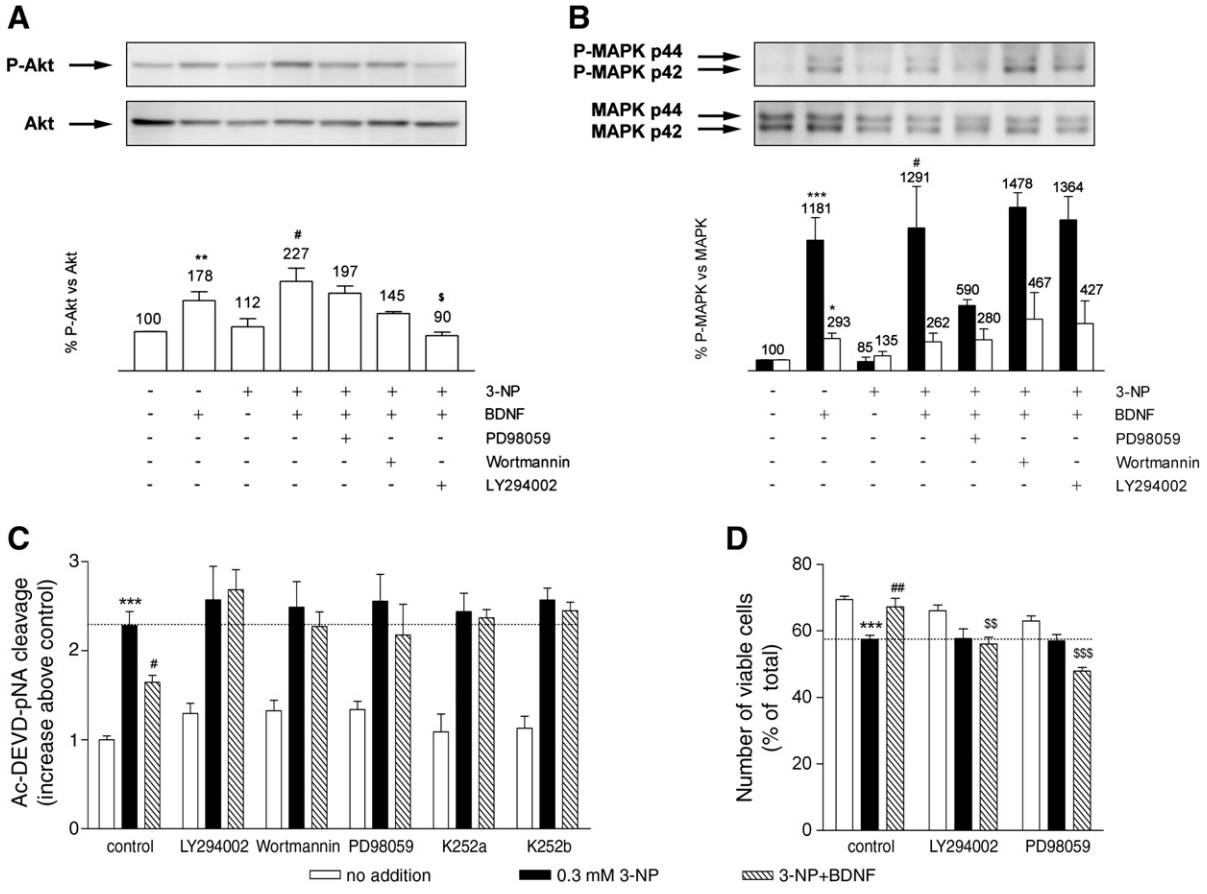

Fig. 2. Activation of PI3K and MEK1/2 signalling pathways upon treatment with BDNF. Cortical neurons were pre-treated with BDNF (100 ng/mL) for 30 min and further incubated in the absence or presence of 0.3 mM 3-NP for 24 h. Before treatment with BDNF, neurons were exposed to LY294002 (5 μM), wortmannin (50 nM), PD98059 (30 μM), K252a (500 nM) or K252b (400 nM) for 1 h. (A, B) Total cell lysates prepared from cortical neurons were analysed by western blotting for phospho-Akt and Akt (A) and phospho-MAPK and MAPK (B). Blots show a representative experiment from 3–4 distinct experiments producing similar results. Graphs show densitometric analysis performed by normalising phosphorylated protein signal to the respective total protein band. (C) Caspase-3-like activity was measured by following the cleavage of the colorimetric substrate Ac-DEVD-pNA. The activity is expressed as the increase of optical density values above the control (untreated neurons). Results are the mean ± SEM of 4–10 distinct experiments. (D) Cells were stained with Hoechst 33342 and assayed for nuclear morphology. Cell viability is expressed as a percentage of the total number of cells (approximately 100 cells per field were counted). Results are expressed as the mean ± SEM of 3 distinct experiments performed in triplicate. Statistical analysis: *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared to untreated neurons; $^{\#}p<0.05$, $^{\#\#}p<0.01$, compared to 3-NP-treated neurons in the absence of BDNF; $^{\$}p<0.05$, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$, compared to 3-NP-treated neurons in the presence of BDNF.

inhibited by wortmannin. In contrast, PD98059, a MEK1/2 inhibitor, decreased MAPK phosphorylation by about 55% in neurons exposed to 3-NP plus BDNF (Fig. 2B), but did not affect Akt phosphorylation (Fig. 2A). These results show that the PI3K and MEK1/2 pathways are independently activated after 24 h incubation with BDNF in the absence or presence of 3-NP, highly suggesting their involvement in BDNF neuroprotection against 3-NP toxicity. To confirm the involvement of these pathways in BDNF neuroprotection against 3-NP toxicity, we also studied the effect of the two PI3K inhibitors (LY294002 and wortmannin) and the MEK1/2 inhibitor (PD98059), as well as two inhibitors of the Trk receptor tyrosine kinase, K252a and K252b, on 3-NP-mediated caspase-3 activation in the presence of BDNF (Fig. 2C). Interestingly, all the inhibitors blocked the protective effect of BDNF, suggesting that the activation of each of these kinases is necessary to protect neurons from 3-NP toxicity as assessed by caspase-3 activation. In addition, LY294002 and PD98059 abrogated the protective effect of BDNF on 3-NP toxicity as measured by nuclear morphology (Fig. 2D).

BDNF regulates Bim expression levels in 3-NP-treated cortical neurons

As shown previously, 0.3 mM 3-NP triggers the activation of caspase-3 through the mitochondrial-dependent pathway (Almeida et al., 2004). Therefore, we next tested whether BDNF could prevent the release of pro-apoptotic proteins from mitochondria of 3-NP-treated neurons. 3-NP caused the release of both cytochrome *c* and Smac/Diablo from mitochondria (Figs. 3A, C), leading to an increase by about 280% ($p<0.001$) and 293% ($p<0.01$) of cytochrome *c* and Smac/Diablo, respectively, in the cytosol (Figs. 3B, D). Treatment with BDNF significantly abrogated the release of both cytochrome *c* ($p<0.01$) and Smac/Diablo ($p<0.05$) from mitochondria. Although not statistically significant, the alterations observed in the mitochondrial fraction for both cytochrome *c* and Smac/Diablo were correlated with the alterations observed in the cytosolic fraction. In the case of cytochrome *c*, its percentage in the mitochondrial and cytosolic fractions was similar to the values obtained in our previous work (Almeida et al., 2004). We also analysed the release of AIF from

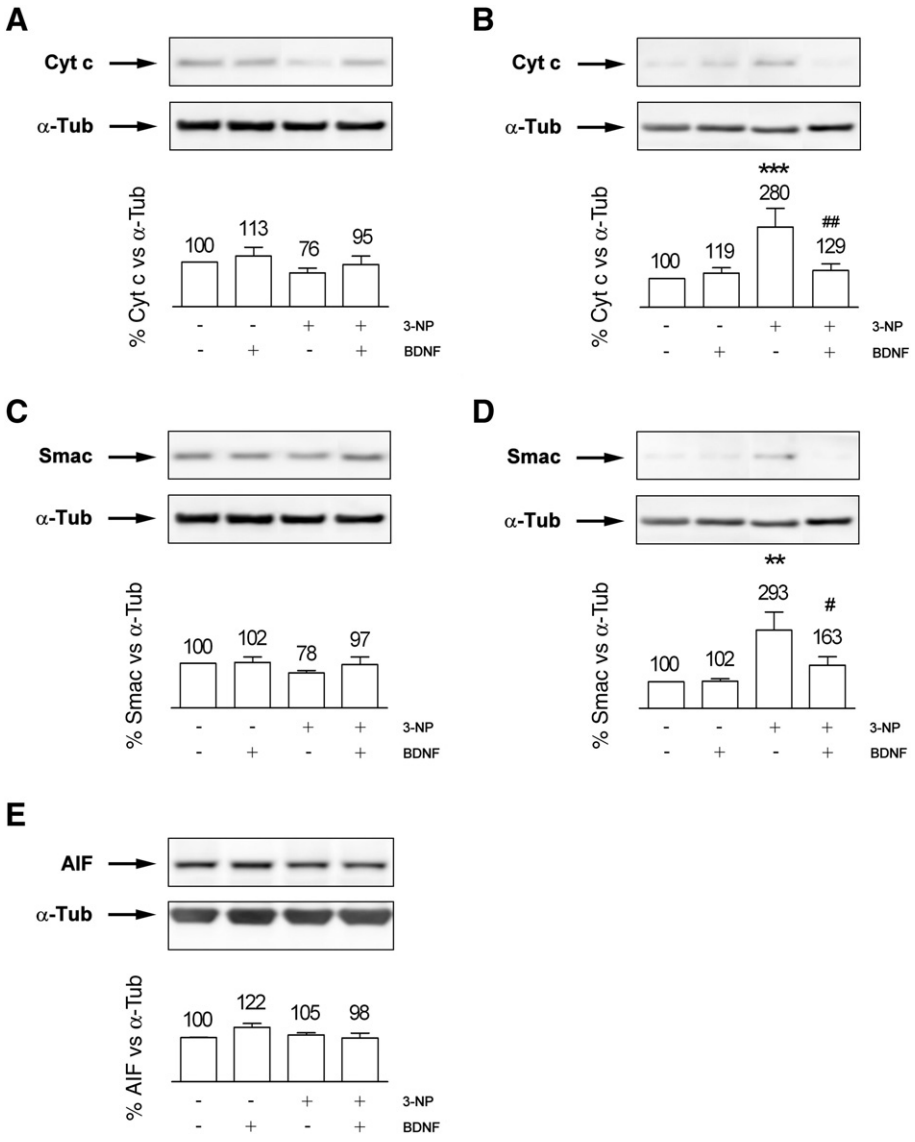

Fig. 3. BDNF abrogates the release of mitochondrial proteins induced by 3-NP. Cortical neurons were pre-treated with BDNF (100 ng/mL) for 30 min and further incubated in the absence or presence of 0.3 mM 3-NP for 24 h. Mitochondrial (A, C, E) and cytosolic (B, D) fractions were prepared from cortical neurons and analysed by western blotting for cytochrome *c* (A, B), Smac/Diablo (C, D) and AIF (E). Blots are representative from 3–5 distinct experiments producing similar results. Graphs show densitometric analysis performed by normalising each protein signal to the α-tubulin (α-Tub) band. Data are the mean ± SEM of 3–5 independent experiments. Statistical analysis: $^{**}p<0.01$, $^{***}p<0.001$, compared to untreated neurons; $^{\#}p<0.05$, $^{\#\#}p<0.01$, compared to 3-NP-treated neurons in the absence of BDNF.

mitochondria in the presence of 3-NP. Although we previously demonstrated that treatment of cortical neurons with 1 mM 3-NP decreased mitochondrial AIF levels (Almeida et al., 2006), the lower concentration of 3-NP (0.3 mM) did not trigger the release of AIF from mitochondria (Fig. 3E).

To further elucidate the mechanism by which BDNF prevents the release of apoptotic proteins from mitochondria and consequently the activation of caspase-3, we evaluated whether BDNF altered the levels of Bcl-2 and Bim, two proteins known to regulate the release of pro-apoptotic proteins from mitochondria (Cory and Adams, 2002). Under the conditions tested, no alterations in mitochondrial or total Bcl-2 levels were observed (Figs. 4A, B), suggesting that the protective effects observed in the presence of BDNF were not dependent on changes in Bcl-2 levels. However, Bim levels in the mitochondria decreased about 30% by BDNF in the absence or presence ($p<0.05$) of 3-NP (Fig. 4C). Interestingly, BDNF also decreased total Bim levels by about 36% in the absence ($p<0.05$) or presence ($p<0.01$) of 3-NP (Fig. 4D). 3-NP treatment alone did not significantly change Bim levels in either mitochondrial or total fractions. BDNF-mediated Bim decrease in the presence of 3-NP was completely blocked by the MEK1/2 inhibitor, PD98059 (40 μM), whereas the PI3K inhibitor, LY294002, did not significantly affect BDNF-mediated Bim decrease in total cell lysates (Fig. 4D). Because the activation of the Ras/MAPK pathway was previously shown to regulate the degradation of Bim (Ley et al., 2003; Meller et al., 2006), we next tested whether BDNF was able to increase Bim turnover. Neurons were incubated with a high concentration of cycloheximide (10 μM), a protein synthesis inhibitor, in the absence or presence of BDNF and/or 3-NP for 2–12 h, and Bim protein levels were measured by western blotting (Fig. 5). The half-life ($t_{1/2}$) of Bim in untreated cortical cultures following cycloheximide treatment was 2.3 h, a value similar to the half-life found by other authors for these cultures (2.8 h; Meller et al., 2006). In the presence of BDNF or BDNF plus 3-NP, the decrease in Bim expression to basal levels was faster, with half-lives of about 0.5 h and 0.8 h, respectively. Interestingly, although 3-NP treatment alone did not significantly change Bim levels (Fig. 4) it delayed the normal degradation of the protein ($t_{1/2}$ ~ 3.9 h). These data show that the decrease in Bim levels following BDNF treatment is due to active degradation of Bim.

Discussion

Striatal neurodegeneration has been shown as a major feature in HD. However, brain imaging studies of HD patients showed that cortical sensorimotor degeneration occurs before motor symptoms in the disease (Rosas et al., 2002, 2005). Consistent with this, it has been proposed that alterations in the cerebral cortex of HD patients reduce cortico-striatal trophic support, leading to striatal degeneration (Zuccato et al., 2001). These results highlight the importance of studying the molecular mechanisms underlying cortical dysfunction and developing therapeutic strategies aimed at preventing cortical and, consequently, striatal neuronal death.

Chronic mitochondrial abnormalities have consistently been reported in HD patients (e.g. Beal, 1992) and the 3-NP animal model used to mimic such alterations. Interestingly, chronic systemic administration of 3-NP in rats and non-human primates produces homogeneous inhibition of complex II (SDH) within the brain and not solely the striatum (Brouillet et al., 1998; Bizat et al., 2003), despite evident striatal degeneration associated with behavioural abnormalities that are reminiscent of HD (Beal et al., 1993; Brouillet et al., 1995). Complementing these reports, rats treated with 3-NP showed reductions in mitochondrial complex I activity in the cerebral cortex, in

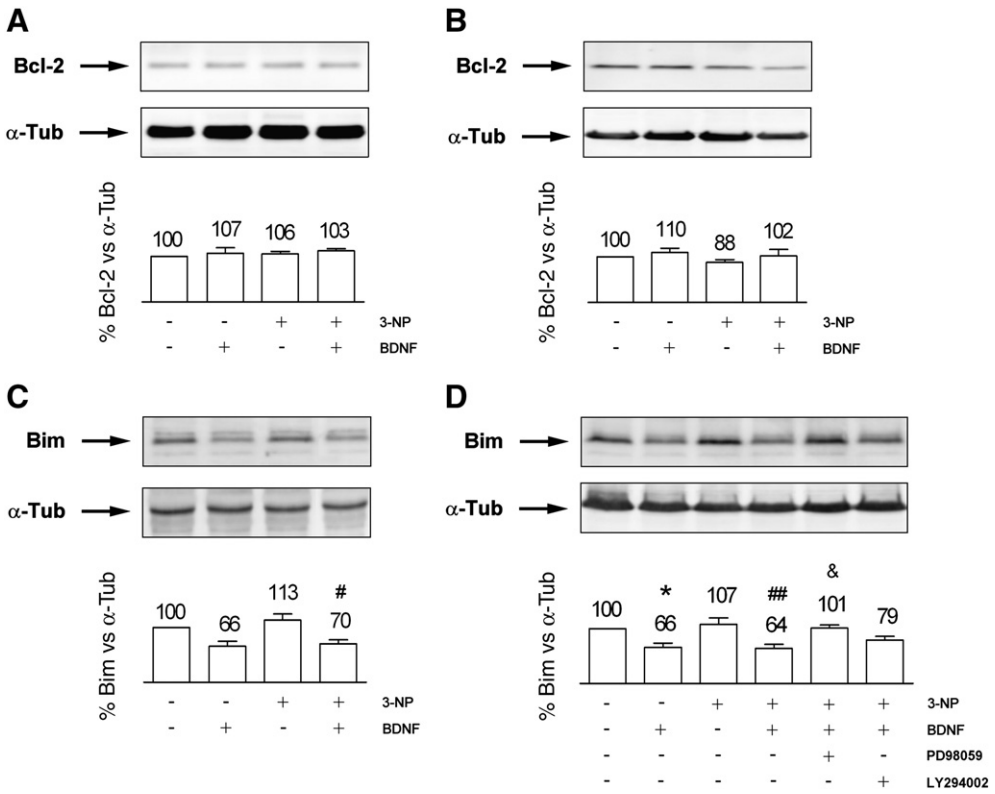

Fig. 4. BDNF decreases Bim protein levels in 3-NP-treated neurons. Cortical neurons were pre-treated with BDNF (100 ng/mL) for 30 min and further incubated in the absence or in the presence of 0.3 mM 3-NP for 24 h. Before treatment with BDNF, neurons were exposed to LY294002 (5 μM) or PD98059 (40 μM) for 1 h. Mitochondrial (A, C) and total cell lysates (B, D) were analysed by western blotting for Bcl-2 (A, B) and Bim (C, D). Blots show a representative experiment from 4–6 distinct experiments producing similar results. Graphs show densitometric analysis performed by normalising each protein signal to the α-tubulin (α-Tub) band. Data are the mean ± SEM of 4–6 distinct experiments. Statistical analysis: $^{*}p<0.05$, compared to untreated neurons; $^{\#}p<0.05$, $^{\#\#}p<0.01$, compared to 3-NP-treated neurons in the absence of BDNF; $^{\&}p<0.05$, compared to 3-NP-treated neurons in the presence of BDNF.

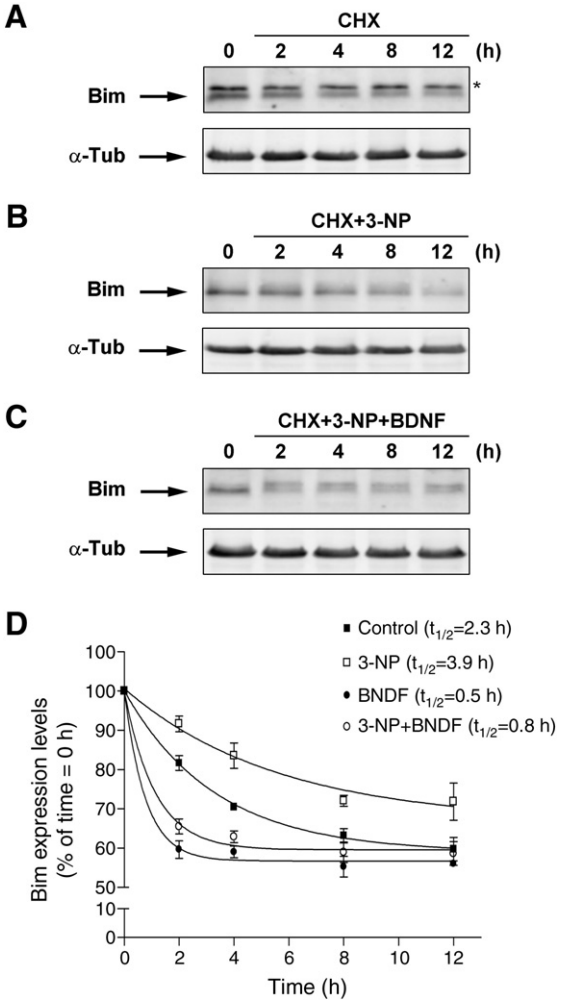

Fig. 5. Effects of 3-NP and BDNF on Bim turnover. Neurons were incubated with cycloheximide (CHX, 10 μM, 1 h pre-treatment) in the absence or presence of BDNF and/or 3-NP for 2–12 h, and Bim levels were determined by western blotting. (A–C) Representative blots, from three distinct experiments producing similar results are shown. (D) Data obtained from the densitometric analysis, performed by normalising each protein signal to α-tubulin (α-Tub), were plotted and fitted to a one-phase exponential decay curve to determine the half-life of Bim (shown in the graph). Data are the mean ± SEM of three distinct experiments. The upper band (*) visible in panel A appears to be non-specific and disappears in subsequent uses of the Bim antibody.

addition to the irreversible inhibition of complex II and SDH activity, which underlie 3-NP-induced cortico-striatal lesion (Pandey et al., 2008). Thus, the cerebral cortex is implicated as an important target for 3-NP-mediated neuronal dysfunction.

We show that BDNF protects cortical neurons from 3-NP-induced release of apoptotic proteins from mitochondria, caspase-3 activation and chromatin condensation/fragmentation. This protection from apoptotic-like cell death is dependent on the activation of PI3K and MEK1/2 pathways. Our results also demonstrate that BDNF increases Bim turnover, leading to a reduction in both total and mitochondrial Bim levels in a process that is mediated by activation of the MEK1/2 pathway.

In this study we were mainly interested in studying mild 3-NP neurotoxicity, as previously shown (Almeida et al., 2004). Therefore, we used 3-NP at a concentration that while decreasing SDH activity by approximately 75% did not affect the activity of the other mitochondrial complexes or ATP levels. Indeed, maintenance of cellular ATP levels is important for apoptotic cell death. Our results show that BDNF treatment protects cortical neurons from 3-NP-induced activation of caspase-3, and consequently apoptosis, through the activation of Trk-dependent PI3K and MEK1/2 signalling pathways. Interestingly, as shown by our inhibitor studies, suppression of caspase-3 induction is maximal when both PI3K and MEK1/2 pathways are activated. Because caspase-3 activation induced by 3-NP most likely resulted from the release of cytochrome *c* and Smac/Diablo from mitochondria, we also evaluated the effect of BDNF treatment on the activation of the mitochondrial-dependent pathway. Our data show that BDNF decreases total Bim levels as well as mitochondrial-localized Bim. Although we did not detect any alteration in Bcl-2 levels, the fact that there is less Bim in the mitochondria after BDNF treatment suggests that more Bcl-2 is available to bind and block Bax and/or Bak oligomerization and the induction of cell death. In support of this, BDNF prevented the release of cytochrome *c* and Smac/Diablo from the mitochondria and consequently the activation of the mitochondrial apoptotic pathway.

The expression levels of Bim are known to be regulated by both the PI3K/Akt and MEK/MAPK pathways. One mechanism involves the phosphorylation of the forkhead transcription factor by Akt, leading to its cytoplasmic retention by 14-3-3 proteins and a resultant loss of Bim gene activation (Brunet et al., 1999). In a second signalling cascade, Bim phosphorylation by MAPK promotes its subsequent ubiquitination and degradation (Ley et al., 2003). Here we show that BDNF-induced reduction of Bim is dependent on MAPK activation and independent from the activation of the PI3K/Akt pathway, since only PD98059 (a MAPK inhibitor) blocked Bim reduction. Similar results were obtained in neuroblastoma cells exposed to paclitaxel (Li et al., 2007). In these cells, and similar to the present study, BDNF treatment reduced Bim levels in a process regulated by the MAPK pathway and not the PI3K/Akt pathway, while both MAPK and PI3K pathways were involved in BDNF protection of neuroblastoma cells from paclitaxel-induced cell death. In differentiated SH-SY5Y cells, BDNF also decreased the levels of Bim by activation of the MAPK signalling pathway (Zhu et al., 2004).

Our results also support the hypothesis that BDNF affects Bim protein stability by increasing its degradation. The half-life of Bim decreased from 2.3 h to 0.5 h in the presence of BDNF and to 0.8 h in the presence of BDNF plus 3-NP. Interestingly, although 3-NP treatment alone did not significantly change the total Bim expression levels, it delayed the basal degradation of the protein ($t_{1/2}$ ~3.9 h). Previously, Luciano et al. (2003) showed that Bim phosphorylation is a prerequisite for ubiquitination. Thus, one possible explanation for the delayed degradation of Bim in the presence of 3-NP could be the activation of phosphatases, which would decrease Bim phosphorylation and, consequently, ubiquitination and degradation. Interestingly, there is also evidence that Bim levels are affected by the presence of mutant huntingtin in HD. Recently, it was reported that knock-in mutant STHdhQ111 striatal cells exhibit significantly increased levels of Bim compared to wild type STHdhQ7 cells (Kong et al., 2009). In addition, increased Bim expression levels were found in the brains of 12-week-old R6/2 mice and 30-week-old R6/1 mice (Zhang et al., 2003; García-Martínez et al., 2007). In their study, García-Martínez et al. (2007) showed that only Bim protein and not mRNA levels were enhanced in R6/1 mice, which suggested that mutant huntingtin may lead to increased Bim levels by interfering with its degradation. One possible mechanism by which mutant huntingtin may decrease Bim degradation is through inhibition of BDNF expression (Zuccato et al., 2001). This would result in a decreased activation of the MAPK pathway and resultant Bim phosphorylation. A second possible mechanism would involve the activation of phosphatases by mutant huntingtin, leading to reduced Bim phosphorylation and, subsequently, reduced ubiquitination and degradation of the protein. In support of this hypothesis, knock-in mutant STHdhQ111 striatal cells were recently shown to be more sensitive to cell death due to high levels of calcineurin activation (Xifró et al., 2008).

In summary, our results demonstrate that BDNF plays an important role in the protection of neurons against neurode-

generation caused by mitochondrial dysfunction due to complex II inhibition. We also found that both PI3K and MAPK pathways seem to be involved in BDNF neuroprotection and that increased Bim degradation underlies BDNF prevention through the MAPK pathway. These findings may be of particular interest in HD since BDNF levels are reduced in the cerebral cortex and striatum of HD patients. Moreover, our data may help to elucidate the protective role of BDNF in cortical neurodegeneration linked to mitochondrial abnormalities.

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