Erratum

BDNF splice variants from the second promoter cluster support cell survival of differentiated neuroblastoma upon cytotoxic stress

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There was an error published in the ePress version of the article *J. Cell Sci*. **122**, 36-43.

The author's names were wrongly given as Baj Gabriele and Tongiorgi Enrico. The correct names appear in the print and online versions of the article.

We apologise for this mistake.

BDNF splice variants from the second promoter cluster support cell survival of differentiated neuroblastoma upon cytotoxic stress

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Summary

The neurotrophin brain-derived neurotrophic factor (BDNF) is a key survival factor for neural cells. In particular, in neuroblastoma tumour cells, expression of the BDNF/TrkB autocrine signalling system promotes a more malignant phenotype and resistance to chemotherapy. The human *BDNF* gene contains two clusters of upstream exons encoding the 5′UTR (exon 1 to exon 3 and exon 4 to exon 9a), these are alternatively spliced to a common exon 9, which contains the coding region and the 3′UTR. At least 34 different *BDNF* mRNA transcripts can be generated, although their physiological role is still unknown. The purpose of this study is to determine which BDNF transcript is involved in cell survival of the human neuroblastoma cell lines SH-SY-5Y (single-copy *MYCN*) and SK-N-BE (amplified *MYCN*). Expression of human *BDNF* mRNAs encoding all possible isoforms was characterised in the two neuroblastoma cell lines. We then investigated whether selective silencing of the different *BDNF* mRNAs using specific siRNAs could reduce cell survival in response to serum deprivation or the anticancer drugs cisplatin, doxorubicin and etoposide. We found that three isoforms located in the second exon cluster are essential for neuroblastoma cell survival under cytotoxic stress. Notably, promoters of the second exon cluster, but not the first, are controlled by Ca^{2+}-sensitive elements.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/122/1/36/DC1

Key words: Brain-derived neurotrophic factor, TrkB, Neuroblastoma, Drug resistance, Serum deprivation, SH-SY-5Y

Introduction

The neurotrophin brain-derived neurotrophic factor (BDNF), is known to regulate a large spectrum of developmental processes of the nervous system, including cell survival, growth and differentiation (Casaccia-Bonnefil et al., 1999; Bibel and Barde, 2000; Huang and Reichardt, 2003). In transformed cells, BDNF can promote differentiation and cell survival even under conditions of cytotoxic stress as in the case of neuroblastoma, a tumour derived from the neural crest that is responsible for at least 15% of cancer-related deaths in children (Brodeur et al., 1992; Matthay, 1995).

A large number of poor prognosis neuroblastoma tumours consistently express BDNF and differentially express the gene encoding its tyrosine kinase TrkB receptor (Brodeur et al., 1992; Nakagawara et al., 1994; Brodeur, 1995). By contrast, neuroblastoma tumours with a good prognosis typically produce high levels of TrkA, the signal-transducing receptor for nerve growth factor (Nakagawara and Brodeur, 1997; Yamashiro et al., 1997). In addition, a subset of neuroblastoma tumours with extremely aggressive growth potential has been shown to be characterised by amplified levels of N-myc (reviewed by Schwab, 2004).

N-myc belongs to the Myc/Max/Mad family of proto-oncogenes encoding transcription factors of the basic-helix-loop-helix-zipper (bHLHZ) class, which regulate the expression of several genes by binding to E-box sequences (Grandori et al., 2000). Early in embryogenesis, N-myc is present primarily in migrating neural crest cells where it promotes cell division and inhibits cellular differentiation; however, later in development, production of N-myc becomes limited to those cells that are undergoing neuronal differentiation, and has a determinative role on the neuronal/non-neuronal choice (Wakamatsu et al., 1997). In neuroblastoma cells, the level of N-myc production correlates with cell growth (Schweigerer et al., 1990) and invasiveness (Goodman et al., 1997), and has a more general effect on the activation of genes involved in protein biosynthesis (Boon et al., 2001; Schwab, 2004).

Studies performed independently by various groups have shown a significant correlation between amplified N-myc and advanced stages of the disease; for example, amplified N-myc was observed in 24 of 48 (50%) stage 3 and stage 4 tumours, but was not detected in 15 stage 1 and stage 2 tumours (Brodeur et al., 1984). However, N-myc amplification is considered highly prognostic of disease progression and outcome independently of disease stage. Indeed, in the absence of amplified N-myc, overall survival is ~60% over a 5 year period, but only 10% of patients survive for 1 year when the gene encoding N-myc (*MYCN*) is amplified to more than ten copies (Seeger et al., 1985).

Previous studies suggested that the BDNF/TrkB autocrine or paracrine signalling pathway is particularly important for survival and metastatic behaviour of aggressive neuroblastoma tumours with N-myc amplification. These studies reported increased survival to cytotoxic drugs in neuroblastoma cells transfected with exogenous TrkB in presence of exogenous BDNF (Middlemas et al., 1999; Ho et al., 2002; Jaboin et al., 2002). However, only one study used anti-BDNF blocking antibodies to determine the role of endogenous BDNF in resistance to serum-free conditions of retinoic-acid-differentiated SH-SY-5Y cells, a neuroblastoma cell line with a single copy of *MYCN* (Feng et al., 2001). Thus, a comprehensive

assessment of the role of endogenous BDNF in protecting neuroblastoma tumours from cell death induced by chemotherapy is still lacking.

BDNF is the result of translation of at least 34 mRNA transcripts produced by alternative splicing of 11 upstream exons (exon 1-9a) (see Fig. 1), each coding for the 5′ untranslated region (5′UTR), spliced to a common downstream exon 9 that encodes the protein and two different 3′UTR sequences (Pruunsild et al., 2007). In human primary neuroblastomas, expression of *BDNF* exon 2, exon 4, exon 6 and exon 9a was found to be significantly more prominent in tumours with an unfavourable prognosis (Aoyama et al., 2001). However, the significance and the specific role of these splice variants in neuroblastoma cell survival is still unknown.

Here we investigated the possibility that selective and transient silencing of BDNF isoforms with short-interfering siRNAs specifically affect cell survival of retinoic-acid-differentiated or undifferentiated neuroblastoma cells in the presence of proapoptotic stimuli. Consequently, we used two neuroblastoma cell lines: SH-SY-5Y with a single copy of *MYCN* and the SK-N-BE with more than 100 copies of the gene.

Results

Retinoic-acid-induced differentiation increases BDNF and TrkB expression in SK-N-BE and SH-SY-5Y cells

Previous studies demonstrated that neuroblastoma cells showed a higher resistance to cytotoxic drugs when TrkB expression was increased either by differentiation (Middlemas et al., 1999) or exogenous expression (Jaboin et al., 2002; Jaboin et al., 2003). Maximal TrkB expression in SH-SY-5Y cells occurs after 5-6 days of retinoic acid treatment (Encinas et al., 2000) but no data are available on SK-N-BE cells. In addition, differentiation increases BDNF expression in SH-SY5Y cells (Garzon and Fahnestock, 2007), but again no information is available on SK-N-BE cells. To assess the effects of differentiation on BDNF and TrkB production in SK-N-BE and SH-SY-5Y neuroblastoma cells, cell cultures were treated with 5 μM 9-*cis* retinoic acid for up to 9 days. Specificity of the TrkB antibody was demonstrated in western blot experiments through detection of the appropriate band in homogenates from rat brain or HEK293T cells transfected with TrkB, and the lack of a signal in homogenate from untransfected HEK293T cells (Fig. 2A, top left). TrkB protein expression in SK-N-BE and SH-SY-5Y cells after 1, 2, 4, 5, 7 and 9 days of retinoic acid treatment was quantified by densitometric analysis of western blots. The dense protein band stained by Ponceau S that appeared at ~45 kDa was used as a control to correct for errors in sample loading. Ponceau S staining has been shown to correlate well with staining for β-actin and GAPDH, and allows for accurate quantification of the amount of total protein bound to a membrane (Olesen and Auger, 2005; Klein et al., 1995). TrkB protein production in differentiated SK-N-BE cells showed a biphasic expression curve (Fig. 2A), with a first peak at 4 days in vitro ($P<0.01$ with respect to undifferentiated cells) and a second, of comparable intensity, at 9 days, with lower values at 5 and 7 days ($P<0.01$ compared with all other time points). By contrast, SH-SY-5Y cells showed a progressive increase from 1 to 5 days and remained stable until 9 days (Fig. 2A) ($P<0.01$, day 2 compared with day 1; NS between day 2, day 4 and day 5; all $P<0.01$ compared with undifferentiated cells). The time course of *BDNF* mRNA expression

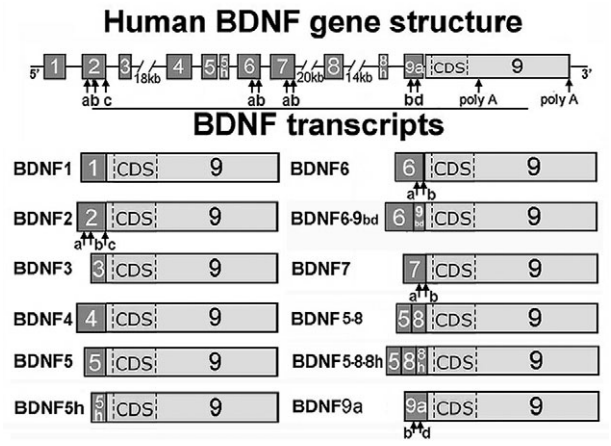

Fig. 1. Schematic representation of human *BDNF* gene structure and its splicing variants. Open boxes represent exons. Lines connect two exons. Arrows indicate alternative polyadenylation sites (PolyA) in the 3′UTR and internal alternative splice sites in exons 2, 6, 7 and 9a (letters a, b, c and d). Exons are drawn to scale.

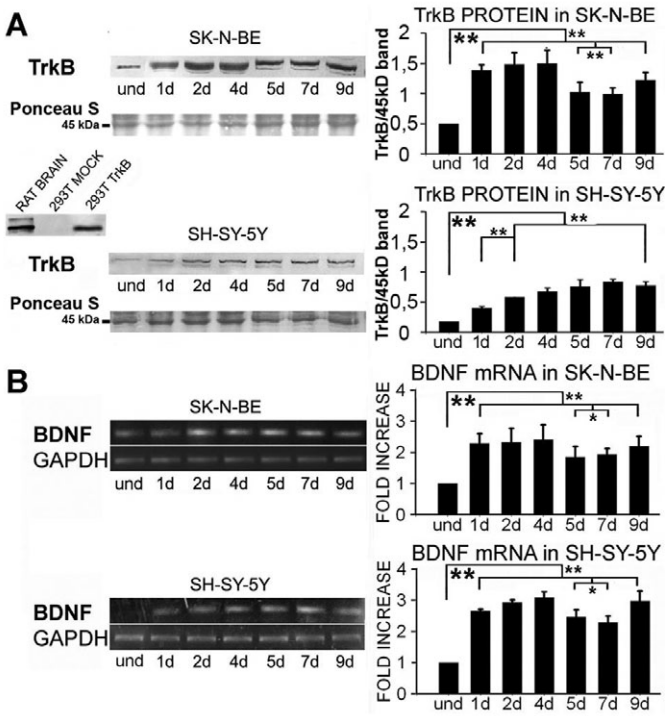

Fig. 2. Retinoic acid differentiation increases production of TrkB protein and *BDNF* mRNA in human neuroblastoma cell lines SK-N-BE and SH-SY-5Y. (A) Western-blot analysis of TrkB expression in SK-N-BE and SH-SY-5Y cell lines at different days (d) of treatment with 5 μM retinoic acid (left; und=undifferentiated). Specificity of the anti-TrkB antibody was tested on rat brain homogenates and on untreated HEK293T cells, which do not express TrkB (negative) or in HEK293T cells transfected with rat full-length TrkB (positive). Densitometric quantification of protein expression revealed a biphasic expression curve in SK-N-BE cells and a progressive increase in SH-SY-5Y cells from day 1 to day 9 (each column represents the mean fold increase with respect to undifferentiated cultures ± s.e.m. from three immunoblots from three different cultures). The Ponceau-S-stained protein band at 45 kDa was used for normalisation. (B) Biphasic expression of *BDNF* mRNA in SK-N-BE and SH-SY-5Y cell lines and densitometric quantification of PCR reactions normalised to expression of *GAPDH* (each column represents the mean fold increase with respect to undifferentiated cultures ± s.e.m. from $n=3$ cDNAs from three different cultures). $^{**}P<0.01$; $^{*}P<0.05$, one-way ANOVA.

parallels that of TrkB, showing a similar biphasic curve in the two cell lines (Fig. 2B). Accordingly, in both cells lines the expression raised significantly at day 1 (P<0.01 vs undifferentiated cells), then remained stable until day 4 (NS difference between day 1, day 2, day 4 and day 9) and showed a slight decrease at day 5 and day 7 (P<0.05). Since the gross morphology of retinoic-acid-differentiated SH-SY-5Y and SK-N-BE cells after 5 days was identical to that at 9 days, the 5-day time point was used for subsequent investigations.

Differentiation increases resistance to cytotoxic stress

We also tested whether differentiation with retinoic acid for 5 days was able to protect neuroblastoma cell cultures from drugs known to induce cell death, such as cisplatin (3 μg/ml), etoposide (3 μg/ml) and doxorubicin (30 ng/ml). Undifferentiated SK-N-BE cells showed a residual viability of ~60% after 24 hours of treatment (Fig. 3A,C) with cisplatin (64.0±6.7%; mean ± s.e.m.), etoposide (61.3±4.1%) or doxorubicin (57.7±4.8%) in the presence of serum. Differentiation with retinoic acid induced a higher resistance to the three cytotoxic drugs compared with untreated SK-N-BE cells (cisplatin 77.7±2.6%; etoposide 75.7±2.3%; doxorubicin 73.1±5.3%. ANOVA vs undifferentiated, P<0.05) (Fig. 3C). Similarly, undifferentiated SH-SY-5Y cells showed a lower residual viability (cisplatin, 44.7±6.5%; etoposide, 47.6±4.6%; doxorubicin, 47.3±3.5%) with respect to retinoic acid-differentiated SH-SY-5Y cells (cisplatin, 62.2±4.0%; etoposide, 58.6±5.2%; doxorubicin, 50.7±3.4%) (Fig. 3D) (ANOVA vs undifferentiated cells, P<0.05 for cisplatin, NS for all other drugs). Thus, neuroblastoma cell lines SK-N-BE and SH-SY-5Y displayed significantly increased protection against cytotoxic drugs after 5 days of retinoic acid treatment. Since the time course of cell death in these conditions has never been reported before, we measured the cell viability of both cell lines during a 48 hour treatment with cisplatin, serum deprivation or cisplatin combined with serum deprivation. The viability curve for the *MYCN* amplified SK-N-BE cells (Fig. 3E), confirmed their greater resistance to all treatments compared with the very sensitive single *MYCN* SH-SY-5Y cells (compare Fig. 3E and 3F). For both cell lines, the effects of serum deprivation and cisplatin were additive (Fig. 3E,F). Interestingly, cultures showed a steep survival curve, rapidly decreasing during the first 12 hours for SK-N-BE [modulus of the slope (m) =13.8] and the first 6 hours for SH-SY-5Y (m=32) (Fig. 3E,F), followed by a more stable viability curve for further time points (m=1.25 for SK-N-BE, m=1.6 for SH-SY-5Y). The change in steepness of the curve is highly significant for both cell lines (P<0.01) and is consistent with a resumption of cell proliferation after an initial blockade. This leads to a phase in which regrowth partially balances the cell loss due to cytoxic stress (data not shown).

Increased resistance to cytotoxic stress depends upon TrkB activation

To show that the survival effect described in Fig. 3 was due to activation of TrkB receptor via secretion of BDNF, we incubated differentiated cells with an siRNA against the coding sequence common to all *BDNF* mRNA transcripts or the Trk inhibitor K252a and measured cell survival in serum-free conditions, as well as the amount of secreted BDNF and activated TrkB-*P* (Fig. 4). Efficacy of the *BDNF* siRNA was tested on SK-N-BE cells transfected with the coding sequence of human *BDNF* fused to GFP. We found a 65% reduction in the number of fluorescent cells 24 hours after transfection, whereas a control siRNA against *GAPDH* had no effect (supplementary material Fig. S1).

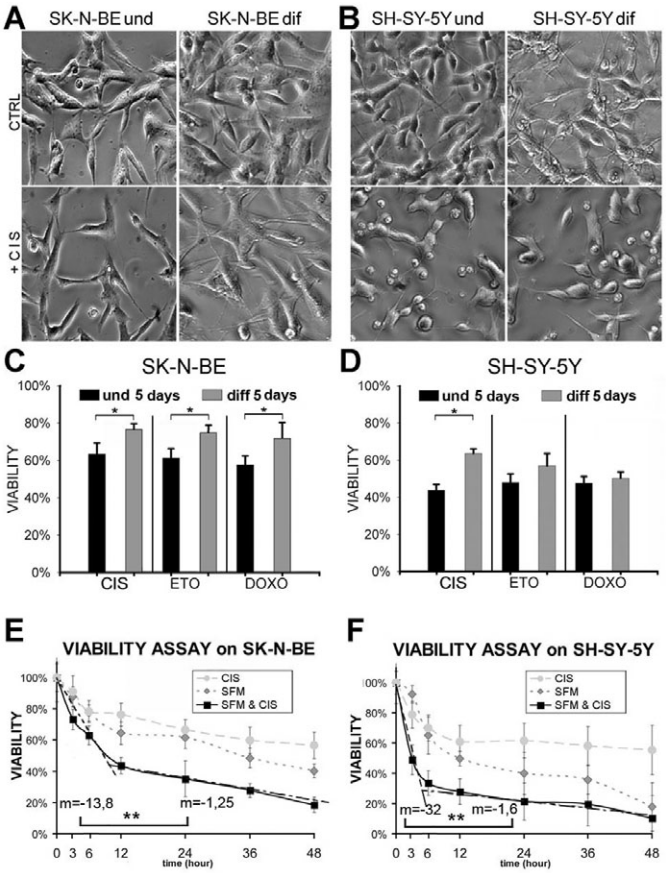

Fig. 3. Retinoic acid differentiation increases viability of neuroblastoma cell lines against cytotoxic drugs. (A) Neuroblastoma cell line SK-N-BE treated with cisplatin (+CIS) in undifferentiated (und) and differentiated (dif) conditions. (B) Neuroblastoma cell line SH-SY-5Y treated with cisplatin (+CIS) in undifferentiated and differentiated conditions. (C) Quantification of the viability assay for SK-N-BE cells in retinoic-acid-differentiated and undifferentiated conditions using the three cytotoxic drugs cisplatin (CIS), etoposide (ETO) and doxorubicin (DOXO). Cells were stained with the nuclear marker Hoechst 33258 and counted (each column represents the mean percentage of survival ± s.e.m.; undifferentiated or differentiated cultures with no drug added were taken as 100%; n=3 cultures). (D) Quantification of the viability assay (n=3) for SH-SY-5Y cells in retinoic-acid-differentiated and undifferentiated conditions using the three cytotoxic drugs as above, performed by counting cells labelled with Hoechst 33258 (mean ± s.e.m.). (E,F) Viability assay of neuroblastoma cell lines under different stress conditions. SH-SY-5Y and SK-N-BE cells were plated and counted at different time points in the presence of cisplatin, in serum-free medium or in serum-free medium (SFM) with cisplatin. Quantification of viability (n=3) for cells was performed with the MTT assay (each column represents mean ± s.e.m.). The survival curves shown in E and F were fitted with linear functions and the corresponding m values are indicated. Statistical analysis was performed with ANOVA (*P<0.05; **P<0.01).

In differentiated cultures, treatment with *BDNF* siRNA in serum-free conditions induced a ~42% reduction in cell survival for SK-N-BE cells and 49% reduction for SH-SY-5Y cells (P<0.01 vs carrier only) measured by MTT assay (Fig. 4A). Inhibition of Trk receptor activation by K252a produced similar effects (44% reduction in SK-N-BE cells, 47% in SH-SY-5Y cells; NS compares with siRNA) (Fig. 4A). The amount of intracellular or secreted BDNF protein in cultures treated with K252a was not significantly different from that of control cultures (SK-N-BE, 250±26 pg/ml lysate, 292±32 pg/ml supernatant; SH-SY-5Y, 212±35 pg/ml lysate;

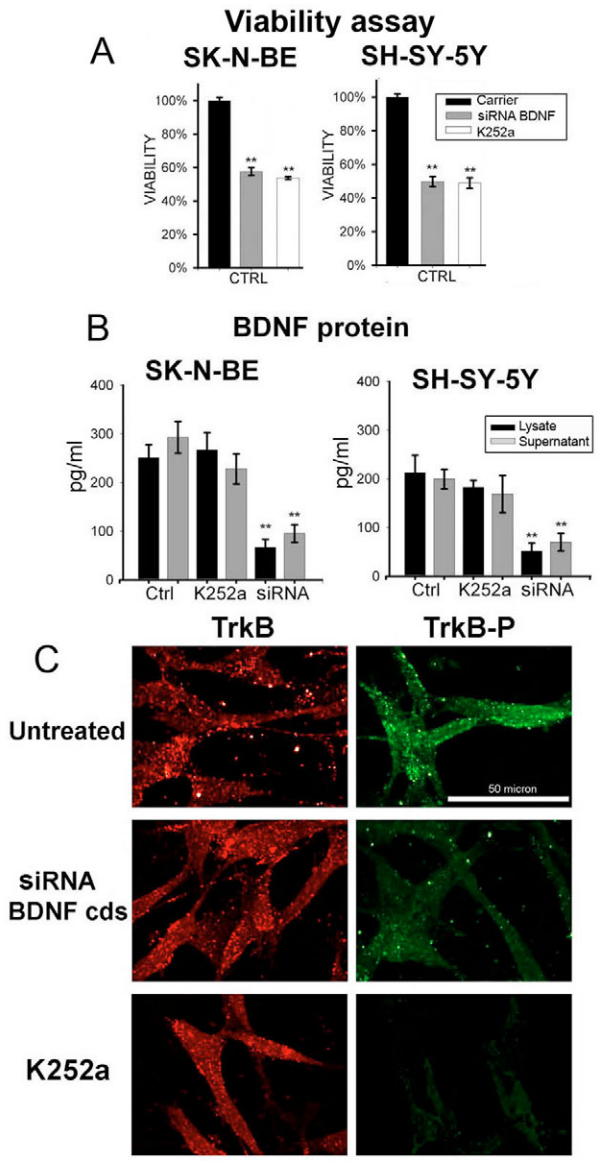

Fig. 4. Increased resistance to cytotoxic stress depends upon TrkB activation. (A) Quantification of the viability (MTT assay) of neuroblastoma cell lines SH-SY-5Y and SK-N-BE treated with *BDNF* siRNA or with the Trk inhibitor K252a. Each column represents mean ± s.e.m. of n=3 independent experiments. (B) BDNF quantification in cell lysates and supernatant of samples treated as in A was carried out by ELISA (Promega). (C) Expression of full-length TrkB, detected by immunofluorescence, was unchanged in SK-N-BE cells treated with *BDNF* siRNA or K252a, whereas activated TrkB, detected with an anti-TrkB-*P* antibody, was markedly reduced by *BDNF* siRNA and completely abolished by K252a. Scale bar: 50 µm. Statistical analysis was performed with ANOVA (**P<0.01).

199±20 pg/ml supernatant). As expected, cultures treated with *BDNF* siRNA showed significantly reduced levels of both intracellular and secreted BDNF protein (SK-N-BE, 74% reduction in lysate, 66% reduction in supernatant; SH-SY-5Y, 76% and 65% reduction in lysate and supernatant, respectively; P<0.01) (Fig. 4B). Accordingly, activation of TrkB was also markedly decreased in cultures treated with either *BDNF* siRNA or K252a as demonstrated by the reduced immunofluorescence obtained using an antibody specific for the phosphorylated form (TrkB-*P*) (Fig. 4C), whereas total TrkB expression remained unchanged (TrkB) (Fig. 4C). Taken together, these results strongly suggest that increased survival induced by differentiation requires BDNF-TrkB signalling pathway activation.

Drug resistance depends upon BDNF expression

To confirm that BDNF expression is required for drug resistance, we measured cytotoxic cell death in neuroblastoma cultures treated with *BDNF* siRNA and cytotoxic drugs (Fig. 5A). Cells treated in normal medium with the three cytotoxic drugs and the transfection vehicle (Fig. 5A, Drugs), showed an average viability of 60%, in agreement with the experiments shown in Fig. 3. SK-N-BE cells treated with *BDNF* siRNA and drugs showed a significantly lower residual survival with respect to drug treatment alone (cisplatin + *BDNF* siRNA, 41.8±3.1%; etoposide + *BDNF* siRNA, 47.6±4.6%; doxorubicin + *BDNF* siRNA 50.3±3.5; ANOVA P<0.01 for cisplatin and doxorubicin and P<0.05 for etoposide) (Fig. 5A). In SH-SY-5Y cells, silencing of *BDNF* expression by siRNA against the coding sequence in the presence of the three drugs produced an effect similar to that observed with SK-N-BE cells; however, results were statistically significant only with cisplatin (residual viability 42%, P<0.05) (Fig. 5B). For this reason, subsequent experiments to investigate the role of single *BDNF* splice variants were carried out only with cisplatin.

To understand whether cytotoxic treatment was able to affect BDNF production, we carried out a comprehensive series of experiments in which we used ELISA to measure the concentration of BDNF in the culture medium (secreted BDNF) as well as in the cell lysate (intracellular BDNF) under the following conditions: no treatment (control), incubation with the Trk inhibitor K252a, siRNA against *BDNF* coding sequence, drug treatment (cisplatin, etoposide and doxorubicin), drug treatment plus K252a, and drug treatment plus *BDNF* siRNA (supplementary material Fig. S2C,D). Treatment with drugs, with K252a or with drugs and K252a did not affect the amount of secreted or intracellular BDNF. By contrast, in neuroblastoma cells treated with *BDNF* siRNA and drugs, we found a downregulation of BDNF protein production that was similar to that observed with siRNA in the absence of drugs (compare Fig. 5C,D with Fig. 4B).

The expression profile of *BDNF* variants in neuroblastomas is more complex than previously reported

Following the demonstration that differentiation increases BDNF-dependent protection against cytotoxic drugs, we decided to investigate the relative contribution of the different splice variants to this protective mechanism. The question is relevant because it was previously reported that splice variants 4, 6 and 9a are preferentially expressed in neuroblastomas with unfavourable biology – those with a greater resistance to cytotoxic drugs and high TrkB expression (Aoyama et al., 2001). However, the biological significance of the expression of these mRNA transcripts was not determined. In addition, novel human *BDNF* mRNAs were recently described in various normal tissues but their expression in neuroblastoma cells was not investigated (Pruunsild et al., 2007).

We initially characterised the *BDNF* mRNA profile of differentiated SK-N-BE and SH-SY-5Y cells according to the novel nomenclature (Pruunsild et al., 2007). Retinoic-acid-treated SK-N-BE cells expressed exon 1, exon 2A, exon 2B, exons 4-8 and exon 9a (i.e. all except exon 3) (Fig. 6A; supplementary material Table S1), whereas differentiated SH-SY-5Y cells expressed only exon 2A, exon 2B, exons 4-6 and exon 9a (Fig. 6A). Thus, the expression

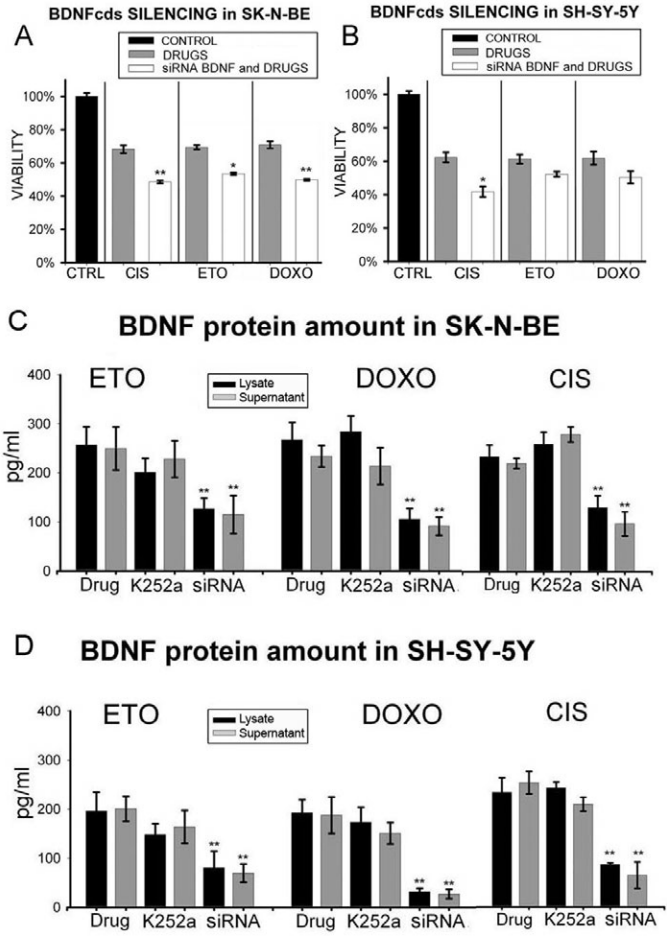

Fig. 5. Silencing of endogenous BDNF reduced resistance of differentiated neuroblastoma cell lines to chemotherapeutic drugs. (A) Cell survival of the neuroblastoma SK-N-BE cell line treated for 24 hours with different drugs, either alone or in the presence of *BDNF* siRNA. Cisplatin (CIS), etoposide (ETO) and doxorubicin (DOXO). (B) Viability of neuroblastoma cell lines SH-SY-5Y co-treated for 24 hours with *BDNF* siRNA and different drugs or with the indicated drugs without siRNA. Quantification of the viability assay for differentiated cells was performed by manually counting the positive cells with respect to total number of cells stained with nuclear marker Hoechst 33258 (each column is the mean ± s.e.m. from n=3 cultures). (C) BDNF quantification in cell lysates and supernatant of SK-N-BE cultures treated with the drug alone, drug + K252a or drug + *BDNF* siRNA was carried out by ELISA (Promega). (D) BDNF quantification in cell lysates and supernatant of SH-SY-5Y cultures treated with the drug alone, drug + K252a or drug + *BDNF* siRNA. Only treatment with siRNA shows a significant reduction in BDNF expression and secretion. Statistical analysis was performed with ANOVA (*P<0.05; **P<0.01).

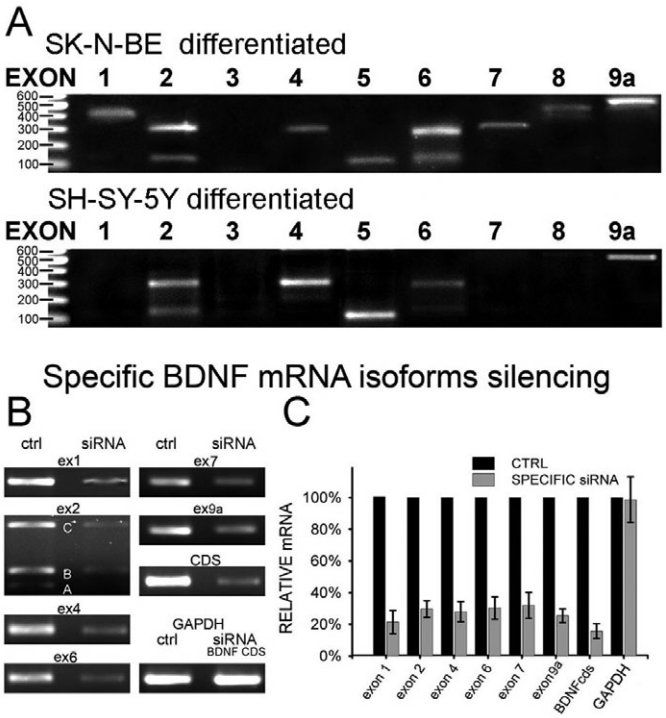

Fig. 6. Expression and silencing efficacy of *BDNF* siRNA isoforms in differentiated SK-N-BE or SH-SY-5Y neuroblastoma cell lines. RNA extraction and cDNA preparation was performed on three different cultures of retinoic-acid-treated cells (5 days with 5 μM retinoic acid) and splice variants were detected by PCR amplification. (A) Differentiated SK-N-BE cultures express the *BDNF* exons 1, 2A, 2B, 4, 5, 6A, 6B, 7, 8 and 9a whereas differentiated SH-SY-5Y cells express exons 2A, 2B, 4, 5, 6B and 9a. (B) Silencing of the different splice variants through siRNA in differentiated SK-N-BE cells. (C) Quantification of silencing efficacy with respect to basal expression in three different cultures (taken as 100%). Silencing efficacy was ranged from 70% to 80%. The housekeeping gene *GAPDH* is not silenced by the *BDNF* siRNA (each column represents the mean ± s.e.m. of three cultures; for quantitative data see supplementary material Table S2).

profile of BDNF variants in the two neuroblastoma cell lines appears more complex than previously reported (Aoyama et al., 2001; Garzon and Fahnestock, 2007).

Survival under conditions of cytotoxic stress requires expression of specific BDNF splice variants

What then is the role of the different BDNF splice variants in cell survival? To answer this crucial question, specific siRNAs against splice variants 1, 2, 3, 4, 6, 7, 9a and the coding sequence (CDS) were generated. The siRNAs to exon 5 and exon 8 could not be generated because of sequence constraints. All siRNAs were tested for their efficacy in differentiated SK-N-BE cells, because this is the cell line and the condition under which the largest number and amount of BDNF variants were expressed (Fig. 6B). The silencing efficiency of the different siRNAs on endogenous *BDNF* mRNA transcripts ranged from 70% to 84% 24 hours after transfection (e.g. 84.3±4.6% for the CDS) (see Fig. 6B,C) (for quantification see supplementary material Table S2). None of the siRNAs affected expression of the housekeeping gene *GAPDH* (Fig. 4B,C).

To determine the role of single *BDNF* mRNA transcripts in neuroblastoma cell survival, we designed a set of experiments that summarised the best experimental conditions found in previous assays. In particular, we used SK-N-BE and SH-SY-5Y cells after day 5 of differentiation with retinoic acid, because most BDNF splice variants were expressed at this time point (see Fig. 6). Second, we used a time period of 24 hours for transfection with the different siRNAs against single BDNF transcripts, because this time was sufficient to demonstrate strong depletion of both *BDNF* mRNA and protein (Fig. 4B; Fig. 6; supplementary material Fig. S1). A previous study in hippocampal neurons showed that the half-life of *BDNF* mRNA is ~2.5 hours (Sano et al., 1996). Third, we measured cell survival following 6 hours of cytotoxic stress (drugs and/or serum-free medium), because this was sufficient time to achieve a 20-70% reduction in cell survival (depending on the conditions),

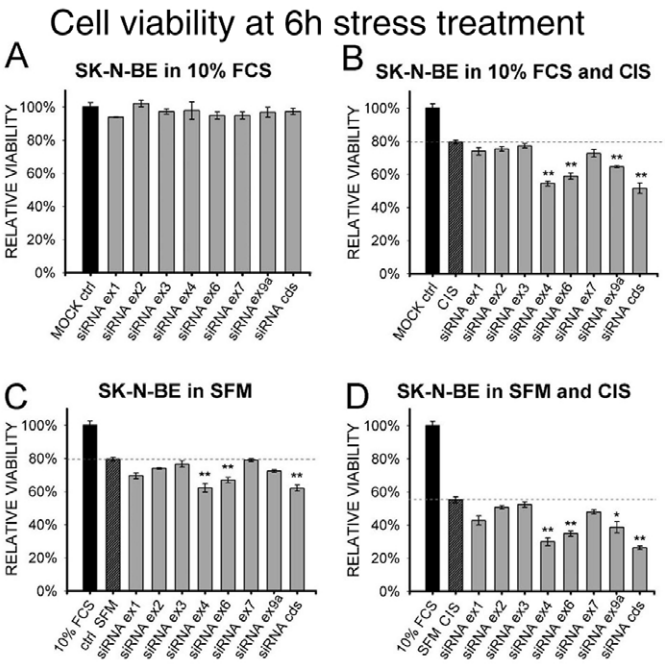

Fig. 7. *BDNF* mRNA transcripts of exon 4, exon 6 and exon 9a are required for resistance to cytotoxic stress conditions. Viability assay using MTT in SK-N-BE cells treated with each single specific siRNA against *BDNF* mRNA isoforms at 6 hours of the following treatments. (A) Control medium with 10% FCS. (B) Control medium with cisplatin (CIS). (C) Serum-free medium (SFM) with no drugs. (D) Serum-free medium (SFM) with cisplatin (CIS). Quantification of the viability assay for cells was performed with MTT (each column is the mean ± s.e.m. of n=3 cultures). Statistical analysis was performed with ANOVA (*P<0.05; **P<0.01).

and the viability curve was characterised by a linear decrease with little or no detectable cell regrowth (Fig. 3). Using these conditions, we first tested whether *BDNF* mRNA transcripts were necessary for survival in differentiated cells in normal culture conditions without environmental stress (10% FCS) and we found that specific silencing of splice variants 1, 2, 3, 4, 6, 7, 9a or the coding sequence did not affect viability of SK-N-BE (Fig. 7A) or SH-SY-5Y cells (supplementary material Fig. S2A). By contrast, in cells treated with cisplatin for 6 hours, specific silencing of exon 4, exon 6, exon 9a or the coding sequence resulted in a significant reduction in survival (P<0.01) (Fig. 7B; supplementary material Fig. S2B) compared with cell survival in the presence of cisplatin only. Notably, silencing of exon 4 or, to a slightly lesser extent, exon 6, produced an effect comparable with that of total *BDNF* silencing with siRNA against the coding sequence. A similar picture, although with slightly higher survival rates, was also obtained in cultures in which the cytotoxic stress consisted of exposure to serum-free medium for 6 hours (Fig. 7C; supplementary material Fig. S2C). Furthermore, with the combination of cisplatin and serum-free conditions for 6 hours, silencing of exon 4, exon 6, exon 9a or the coding sequence caused a significantly lower viability with respect to that observed in cultures treated with serum-free medium and cisplatin without siRNAs (P<0.01 for exon 4 and exon 6; P<0.05 for exon 9a) (Fig. 7D; supplementary material Fig. S2D). In all cases, transfection with siRNA specific to exon 3, which was not detectable in the two cell lines, did not produce any change in viability, supporting the conclusion that the effects observed with the siRNAs against the other exons were specific.

Discussion

We provide here a comprehensive assessment of the role of endogenous *BDNF* gene transcripts in protecting differentiated neuroblastomas from cell death induced by chemotherapy and serum deprivation. In particular, we show here that selective and transient silencing of BDNF isoforms 4 and 6 (and in some cases, also isoform 9a) with siRNAs significantly reduces resistance to cytotoxic treatment of differentiated SH-SY-5Y (with a single copy of *MYCN*) and SK-N-BE (with >100 copies of *MYCN*) neuroblastoma cell lines. Silencing of any splice variants, or even the entire endogenous BDNF protein, did not affect viability of either of the neuroblastoma cell lines when cultured with 10% FCS, a condition that is likely to provide a sufficient amount of exogenous growth factors to support cell survival. Our data also indicate that a high dependence on BDNF for viability is observed only when neuroblastoma cells are exposed to stressful conditions.

Consistently, exogenous BDNF has been shown to increase neuroblastoma survival against chemotherapeutics such as doxorubicin, etoposide and cisplatin or serum-free conditions (Ho et al., 2002; Jaboin et al., 2002). Since BDNF promotes cell survival and protects neuronal cells from injury through an autocrine/paracrine BDNF-TrkB signalling pathway (Feng et al., 2001; Jaboin et al., 2002), one would expect that secretion of endogenous BDNF produced by any single *BDNF* transcript would be sufficient to support cell survival. However, in this study we found that, only exon 4, exon 6 and exon 9a splice variants were needed to protect against acute cytotoxicity. This finding is particularly intriguing and warrants detailed discussion.

In human primary neuroblastomas, expression of *BDNF* exon 2, exon 4, exon 6 and exon 9a was found to be significantly more prominent in primary tumours with unfavourable biology (Aoyama et al., 2001). It must be noted that exon 3, exon 5, exon 7 and exon 8 were not known at the time of the seminal work by Aoyama and co-workers (Aoyama et al., 2001). A recent real-time PCR analysis in SH-SY-5Y cells showed high expression of BDNF exon 1, exon 2, exon 4 and exon 6, with exon 4 and exon 6 accounting for 80% of total *BDNF* mRNA, whereas exon 3, exon 7 and exon 9a were not detectable; exon 5 and exon 8 were not investigated (Garzon and Fahnestock, 2007). Our study overlaps with these two fundamental studies in that exon 2, exon 4, exon 6 and exon 9a are clearly expressed in both SK-N-BE and SH-SY-5Y cell lines. We also found that SK-N-BE neuroblastoma cells, deriving from a more aggressive tumour, also express exon 1, exon 5, exon 7 and exon 8 (in total, all exons but exon 3), whereas SH-SY-5Y cells, in addition to exon 2, exon 4, exon 6 and exon 9a also express exon 5, albeit at much lower levels. The lack of expression of exon 1 and the presence of exon 9a BDNF isoforms in SH-SY-5Y cells shown here represents a discrepancy with respect to the earlier study that might be explained by the different times of differentiation (5 vs 9 days) and culture media used (Garzon and Fahnestock, 2007). The finding that exon 4, exon 6 and exon 9a are needed for neuroblastoma protection against cytotoxicity provides a rationale for the prominence of these transcripts in more aggressive and drug-resistant tumours. What is then special in these transcripts?

In this study, we clearly show that SK-N-BE neuroblastoma cells produce and actually secrete higher amounts of BDNF protein than that observed in SH-SY-5Y cells. However, it remains unclear whether this is due to a higher rate of translation of the particular *BDNF* mRNAs. Unfortunately, information about the ease of translation of the different *BDNF* mRNAs is presently limited to a single study reporting that splice variants 1, 2, 4 and 6 are equally

abundant in a polyribosome-enriched fraction extracted from adult rat brain (Timmusk et al., 1994). Conversely, a large body of data shows that BDNF splice variants are differentially regulated at the transcriptional level. It has been reported that the different promoters of *BDNF* can be grouped in two distinct clusters, with one cluster comprising promoters for exon 1, exon 2 and exon 3 and the other those for exon 4, exon 5, exon 6 and exon 7 (Pruunsild et al., 2007). Promoters of the first cluster (exon 1, exon 2 and exon 3) have a slow transcriptional activity but their expression persists for a long time in response to a variety of stimuli (Metsis et al., 1993; Lauterborn et al., 1996; Nanda and Mack, 1998; Marmigere et al., 2001). By contrast, exons from the second promoter cluster are transcribed as immediate early genes and have a rapid decay (Lauterborn et al., 1996; Nanda and Mack, 1998; Marmigere et al., 2001). Thus, cluster 1 promoters seem to provide the cell with a constitutive amount of *BDNF* mRNA transcripts, and their transcription occurs over a long time scale, whereas exons under cluster 2 promoters seem to provide a more regulated set of transcripts that requires continuous transcription to maintain high levels (Lauterborn et al., 1996; Pruunsild et al., 2007). As treatment of neuroblastoma cells with the transcriptional inhibitor actinomycin D might reduce cell survival under specific conditions (Feng et al., 2001; Wang et al., 2007), we reasoned that continuous production of *BDNF* mRNA transcripts with rapid turnover might be crucial for cell survival. Remarkably, our data provide compelling evidence that viability of neuroblastoma cells under stress conditions depends on production of cluster 2 exon 4, exon 6 and in part, exon 9a isoforms, suggesting that a recurrent transcription of these high-turnover transcripts is crucial to protect neuroblastoma cells from chemotherapy or serum deprivation.

The latter conclusion implies that transcription of cluster 2 promoters should be induced by the conditions of stress described above. The mechanism of action of the three drugs used in the present study is compatible with this model. In fact, although they all target DNA replication by acting upon different mechanisms (Fornari et al., 1994; Hande, 1998; Jordan and Wilson, 2004), the signals generated from chromatin blockade are known to activate apoptosis through a common Ca^{2+}-dependent signalling pathway (West et al., 2002). It now seems accepted that mRNA transcripts arising from cluster-2 promoters, which have regular TATA/CAAT box motifs, can respond quickly to any Ca^{2+} increase (Liu et al., 2005). Indeed, cluster 2 promoters contain transcriptional regulatory elements recognised by Ca^{2+}-responsive transcription factor (CaRF), cAMP/calcium-responsive element binding proteins (CREB) and methyl-CpG binding protein 2 (MeCP2) (Tao et al., 2002; Chen et al., 2003; Martinowich et al., 2003). Of note is the fact that although exon 4, exon 6, exon 8 and exon 9a have a regular TATA/CAAT box, it was not possible to identify clear TATA/CAAT box motifs 5′ to the ends of exon 5 and exon 7 (Alarcon et al., 1999; Liu et al., 2005). Accordingly, our data show that specific silencing of exon 4, exon 6 and exon 9a induce a significant reduction in cell survival under stress conditions, suggesting that the drug resistance could be mediated by the expression of these quickly inducible *BDNF* transcripts. In keeping with this model, it must be also noted that doxorubicin, etoposide and in particular, cisplatin, which have remarkable effects on neuroblastoma cell survival, not only block DNA replication but also have an inhibitory effect on RNA transcription (Fornari et al., 1994; Hande, 1998; Jordan and Wilson, 2004).

In conclusion, the present study provides a novel rationale for the role of *BDNF* mRNA transcripts in cell survival under cytotoxic conditions and, more generally, for the significance of the different *BDNF* transcripts. Upregulated *BDNF* mRNA transcripts encoding exon 4, exon 6 and exon 9a might represent an additional marker for aggressive neuroblastomas. Indeed, tumours with high expression of these *BDNF* transcripts might be less likely to differentiate, regress spontaneously or respond to therapy. Thus, any drug that suppresses transcription and/or translation of these BDNF isoforms could help to improve the therapy of these paediatric tumours.

Materials and Methods

Cell culture and reagents

Human neuroblastoma cell lines SH-SY-5Y and SK-N-BE were grown in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 g/ml streptomycin at 37°C with 5% CO_2. To induce differentiation, 5 μM retinoic acid final concentration was added to cultures at the time of plating, and replaced every 48 hours. Cells were analysed undifferentiated or after 1, 2, 4, 5, 7, 9 days of retinoic acid treatment. Undifferentiated or differentiated (5 days) cultures were treated with 50 ng/ml Cisplatin (Bristol-Myers Squibb), 5 μg/ml Etoposide (Bristol-Myers Squibb), or 50 ng/ml Doxorubicin (Pharmacia). All experiments were performed in triplicate and repeated in three independent assays.

Western blotting

Cells at the different differentiation stages were lysed on ice in lysis buffer [150 mM NaCl, 25 mM HEPES pH 7.4, 1% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate, 1 mM EGTA, 1 mM EDTA and 1 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin and 10 mg/ml pepstatin]. Protein concentration was determined by Bradford assay (Bio-Rad). Protein lysates were submitted to 10% SDS-PAGE (30 μg for SH-SY-5y and 20 μg for SK-N-BE) and electroblotted onto nitrocellulose. Western blots were incubated at 4°C overnight with anti-TrkB antibody (C-13; Santa Cruz) diluted 1:500 in PBS/5% fat-free milk and for a further 1 hour at room temperature with anti-rabbit alkaline-phosphatase-coupled antibody (1:1000; DAKO Cytomation). Membranes were developed at room temperature with 35 mg/ml 4-nitroblue-tetrazolium (NBT; Labtek Eurobio) and 17.5 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Labtek-Eurobio) in 100 mM Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl. Equal protein loading was confirmed by quantification analysis performed using the densitometry protocol of ImageJ software (NIH). For this purpose the western blot membranes were stained with Ponceau S and scanned at high resolution (1200 dpi). Ponceau S staining was obtained by incubating membranes in Ponceau S solution (0.5% Ponceau S, 1% glacial acetic acid in deionised water) for 5 minutes at room temperature with agitation and washed twice for 2 minutes each in distilled water.

Elisa assay for BDNF detection

BDNF production and secretion in SK-N-BE and SH-SY-5Y neuroblastoma cell lines was assessed using the ELISA BDNF Emax Immunoassay System. Lysate and supernatant collected from cells in the same conditions were used for the viability assay and were diluted 1:100 and plated on wells previously coated following the manufacturer's instructions.

Semiquantitative RT-PCR

Total RNA was extracted from whole rat brain using the TriZol® Reagent (Invitrogen) from SH-SY-5Y and SK-N-BE cells at the indicated differentiation stages. Total RNA (1 μg) was reverse-transcribed into cDNA using 200 U SuperscriptIII reverse transcriptase (Invitrogen) and random hexanucleotides. PCR was performed in a 25 μl volume containing 1 μg cDNA, 10× reaction buffer, 500 mM KCl, 100 mM Tris-HCl, pH 9; 1.5 mM $MgCl_2$; 200 μM dNTPs mix; 0.04 U *Taq* DNA polymerase (all from Invitrogen) and specific forward and reverse primers (Proligo) for the different BDNF isoforms (supplementary material Table S1). Quantification analysis was performed using the densitometry protocol of ImageJ software (NIH) and normalising each band with the internal controls.

Preparation of siRNA

The uniqueness of the sequences used to design each siRNA was ascertained by a BLAST search. Primers (see supplementary material Table S2) contained the T7-polymerase sequence at one end (Proligo). PCR products were gel-purified and transcribed using T7-RNA polymerase and the Silencer siRNA mixture kit (Ambion). Following DNase and RNase-A treatment for 1 hour, double-stranded RNA was purified through spin columns (Promega) and digested with RNase III according to the manufacturer's instructions to yield siRNA pools. This methodology allows producing pools of 12-30 bp double-stranded RNAs specific for each single isoform. These RNA duplexes contain 5′-PO_4, 3′-OH, and 2-nucleotide 3′overhangs similar to siRNA produced in vivo. The effect of the siRNA against the coding sequence was verified by cotransfecting neuroblastoma cells with a construct encoding BDNF-CDS in fusion with green fluorescent protein (GFP) and then the number of green

cells was counted and the staining intensity was evaluated by densitometry on single cells (ImageJ software, NIH). Quantification of the silencing assay was also performed by staining the cells with nuclear stain Hoechst 33258 and counting green cells without or with treatment with siRNA (mean ± s.e.m.). In addition, semiquantitative PCR from SK-N-BE differentiated for 5 days was carried out after transfection with siRNA against each single exon. Quantification analysis was performed using the densitometry protocol of ImageJ software (NIH) and normalising each band with the internal GAPDH control.

Cell-survival analysis

A colorimetric MTT assay was performed to measure cell survival. 10,000 SH-SY-5Y cells and 5000 SK-N-BE cells were plated into each well of 96-well plates. Cells were differentiated for 4.5 days and were transfected with 25 ng/well siRNA for each single BDNF exon using Lipofectamine-2000 (Invitrogen). The medium was changed after 24 hours with fresh DMEM with 10% FBS or with serum free DMEM and chemotherapeutic agents were added. Cell number was assessed 6 hours later by MTT assay (Sigma) using a 570-630 nm dual wavelength absorbance protocol in order to reduce instrumental variability and eliminate nonspecific absorption. The steepness of the viability curve was assessed using the slope parameter 'modulus of the slope' (m) in order to detect the correct time point for assay analysis.

Statistical analysis

Data were analysed using the SIGMA-STAT statistical package (SYSSTAT Software, Inc). One-way ANOVAs performed using the Student-Newman-Keuls method were used to determine significant differences ($P<0.01$) between groups.

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