

In Vivo Restoration of Physiological Levels of Truncated TrkB.T1 Receptor Rescues Neuronal Cell Death in a Trisomic Mouse Model Report

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Summary

Imbalances in neurotrophins or their high-affinity Trk receptors have long been reported in neurodegenerative diseases. However, a molecular link between these gene products and neuronal cell death has not been established. In the trisomy 16 (*Ts16*) mouse there is increased apoptosis in the cortex, and hippocampal neurons undergo accelerated cell death that cannot be rescued by administration of brain-derived neurotrophic factor (BDNF). *Ts16* neurons have normal levels of the TrkB tyrosine kinase receptor but an upregulation of the TrkB.T1 truncated receptor isoform. Here we show that restoration of the physiological level of the TrkB.T1 receptor by gene targeting rescues *Ts16* cortical cell and hippocampal neuronal death. Moreover, it corrects resting Ca^{2+} levels and restores BDNF-induced intracellular signaling mediated by full-length TrkB in *Ts16* hippocampal neurons. These data provide a direct link between neuronal cell death and abnormalities in Trk neurotrophin receptor levels.

Introduction

Linking specific genes to developmental or pathological conditions is one of the most challenging tasks in experimental biology. While gene targeting can identify the direct involvement of a gene in a specific developmental process, in pathological conditions, this task is much more complex due to the numerous alterations in gene or protein expression levels that are associated with the disease. Most studies are correlative, and rarely can an unambiguous connection between a gene product and a phenotype be established. One successful example is Huntington's disease, where numerous lines of

evidence supported the notion that a mutated huntingtin fragment was the cause of the disease, and a causal relationship between mutant protein expression and progression of the pathology was shown with a conditional mouse model where the blockade of expression of the mutant protein in symptomatic mice lead to the disappearance of inclusions and the amelioration of the behavioral phenotype (Yamamoto et al., 2000).

Neurotrophins and their receptors are critical for normal nervous system development (Bibel and Barde, 2000; Huang and Reichardt, 2001; Snider, 1994; Tessarollo, 1998). Changes in their expression have been reported in a variety of pathological conditions, including neurodegenerative diseases (Connor et al., 1996; Dawbarn and Allen, 2003; Ferrer et al., 1999). However, while they have been considered targets for clinical intervention, their direct role in causing pathologies of the nervous system is still unclear. Neurotrophin heterozygous mice have been a useful system to investigate the effects of reducing neurotrophin levels in the adult, which include obesity and increased aggression in *BDNF^{+/-}* mice and memory deficits in *NGF^{+/-}* mice (Chen et al., 1997; Lyons et al., 1999). However, to date, there is no information on whether dysregulation of Trk receptor isoforms, often observed in pathological conditions, may affect neuronal physiology (Dawbarn and Allen, 2003; Ferrer et al., 1999). Neurons from the trisomy 16 (*Ts16*) mouse represent a useful model to study the effects of dysregulation of a variety of genes and its relative impact on neuronal survival (Coyle et al., 1991). Ex vivo, hippocampal neurons undergo accelerated cell death that can be rescued by a variety of exogenous factors, including fibroblast growth factor 2 and neurotrophin-3 (Dorsey et al., 2002). However, in these rescue experiments it is difficult to identify the primary defects leading to cell death because pharmacological activation of strong prosurvival pathways could mask the intrinsic cellular defect.

We previously reported that the accelerated death of *Ts16* neurons could be prevented by exogenous NT-3, but not BDNF, a potent autocrine survival factor for normal hippocampal neurons, demonstrating a selective defect in BDNF responsiveness (Dorsey et al., 2002). *Ts16* neurons overexpress the T1 isoform of truncated TrkB (TrkB.T1), and the premature hippocampal neuron cell death could be rescued by overexpression of the full-length, catalytically active isoform of TrkB. Since TrkB.T1 can act as a dominant-negative inhibitor of BDNF signaling via full-length TrkB (Eide et al., 1996), TrkB.T1 overexpression may cause the accelerated death of *Ts16* neurons.

In this study, we used a gene-targeting approach to reduce TrkB.T1 to physiological levels in the *Ts16* mouse in order to test the hypothesis that overexpression of TrkB.T1 is the primary cause of the accelerated *Ts16* hippocampal neuron death in vitro and increased neuronal apoptosis in vivo. We found that genetic reduction of TrkB.T1 expression to a physiological level rescues neuron cell death both in vitro and in vivo. This rescue is associated with a restoration of TrkB and Akt

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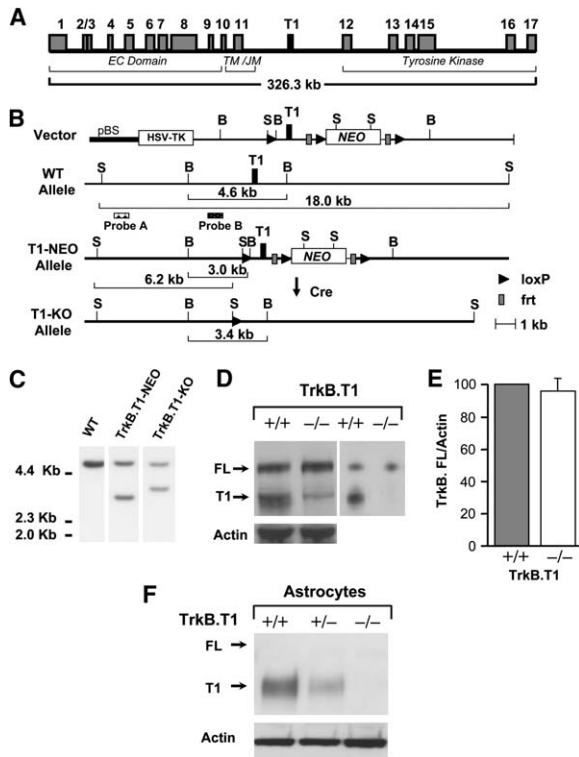


Figure 1. *TrkB.T1* Deletion Does Not Affect Full-Length TrkB Expression

(A) Diagram of the *TrkB* locus showing the position of the *TrkB.T1*-specific coding exon relative to the exons encoding the tyrosine kinase catalytic domain. (B) Schematic representation of the replacement type vector and strategy used to delete the *TrkB.T1* exon. Restriction enzyme sites are as indicated. B, BamHI; S, SpeI. pBS indicates the pBluescript cloning vector. Southern blot analysis of ES cell DNA using an SpeI digest and probe A detected the rearrangement in the mouse *TrkB* locus (not shown). (C) Southern blot analysis of tail DNA from a litter obtained by intercrossing a *TrkB.T1-NEO*^{+/-} mouse with a β -actin *CRE*^{+/+} mouse, using a BamHI digest and probe B shown in panel (A). Note the switch of the 3 kb band (center lane) to 3.4 kb (right lane) after Cre excision of the *TrkB.T1* exon. (D) Specific loss of the *TrkB.T1* isoform in whole-brain lysates prepared from 3-month-old *TrkB.T1*^{-/-} animals. Western blot analysis using the *TrkB*_{out} antibody as described in Experimental Procedures (left panel). The bottom panel shows the blot stripped and reprobed with an antibody to actin. The right panel represents a wheat germ agglutinin precipitation from whole-brain lysate, probed with *TrkB*_{out}. Note the specific loss of the truncated *TrkB* receptor. (E) Ratios of full-length *TrkB* receptors relative to actin in whole-brain lysates are similar in *TrkB.T1*^{+/+} (n = 3) and *TrkB.T1*^{-/-} (n = 3) animals. *TrkB.T1*^{+/+} is set at 100%. Error bars indicate SEM. (F) Targeting of *TrkB.T1* does not affect expression of the *TrkB* kinase receptor in primary astrocyte cultures. Astrocytes were prepared from E16.5 *TrkB.T1*^{+/+}, *TrkB.T1*^{+/-}, and *TrkB.T1*^{-/-} embryos, lysed, and analyzed by Western blot using the *TrkB*_{out} antibody (top panel). The bottom panel shows an immunoblot for actin as a loading control.

phosphorylation in response to BDNF as well as normalization of resting Ca²⁺ levels in neurons. Our results suggest that alterations of receptor isoform expression can affect neurotrophin signaling and consequently neuron survival. Small alterations in neurotrophin/Trk receptor activation like those seen in mouse *Ts16* may be directly linked to neurodegenerative diseases.

Results

Selective Deletion of the *TrkB.T1* Isoform

To decrease the levels of *TrkB.T1*, we first used a gene targeting approach to remove the *TrkB.T1* coding exon, which is located between the juxtamembrane and the tyrosine kinase coding exons (Figure 1). To avoid disruption of the full-length *TrkB* isoform containing the tyrosine kinase, we inserted loxP sites into the genomic regions that showed no conservation between the mouse and human sequence (Figure 1B). We reasoned that a lack of conservation between the two mammalian species indicated that these areas are not important for splicing regulation. The absence of *TrkB.T1* in mutant mice was verified in whole-brain lysates (Figure 1D). Since a *TrkB*-specific antibody raised against the extracellular domain showed the presence of a background band at about the same position on the gel as *TrkB.T1*, we prepared whole-brain wheat-germ lysates and probed with the same antibody. This procedure, which was used to enrich for glycosylated membrane proteins such as Trk receptors, eliminated the background band (Figure 1D, right panel). We next investigated whether elimination of the genomic sequence between the transmembrane and the tyrosine kinase coding exons would affect the expression of the full-length *TrkB* receptor (Figures 1D and 1E). No difference in the level of full-length *TrkB* was found between the *TrkB.T1* mutant and control mice, suggesting that targeting of the *TrkB.T1* truncated isoform did not affect the expression level of full-length *TrkB* (Figure 1E). To investigate whether splicing of the different *TrkB* isoforms was affected by the targeting, we analyzed isoform expression in cultured primary astrocytes, a cell type that expresses only truncated *TrkB* (Rose et al., 2003). Neither full-length *TrkB* nor *TrkB.T1* expression was noted in astrocytes from homozygous *TrkB.T1* mutant mice, suggesting that the genomic elements controlling splicing were not affected. Moreover, we observed a reduction of the *TrkB.T1* receptor level in the heterozygous mutant astrocytes, suggesting a potentially useful strategy to correct *TrkB.T1* levels in the *Ts16* mouse (Figure 1F).

In Vivo Reduction of *TrkB.T1* Restores *Ts16* Hippocampal Neuron Survival

To determine whether reducing *TrkB.T1* levels would rescue *Ts16* hippocampal neurons from premature death, we intercrossed *TrkB.T1* homozygous mice with the Robertsonian *Ts16* parental strain, which is doubly heterozygous for chromosomal translocations 6:16 and 16:17 (Gearhart et al., 1986). The resulting progeny were all heterozygous for *TrkB.T1* and were either euploid or trisomic (monosomic conceptuses are early embryonic lethal). Cultured hippocampal neurons were prepared from littermate euploid and trisomic embryos at day 16.5 (E16.5) and maintained in serum-free medium containing the chemically defined supplement B27. By the second day in vitro, the cultures consisted primarily of postmitotic, differentiated neurons with extensive process formation, as previously described (Dorsey et al., 2002). At 3 days in vitro, we examined the levels of *TrkB.T1* in hippocampal neurons from the *Ts16* mice heterozygous for *TrkB.T1* and found that *TrkB.T1* protein had been reduced to levels

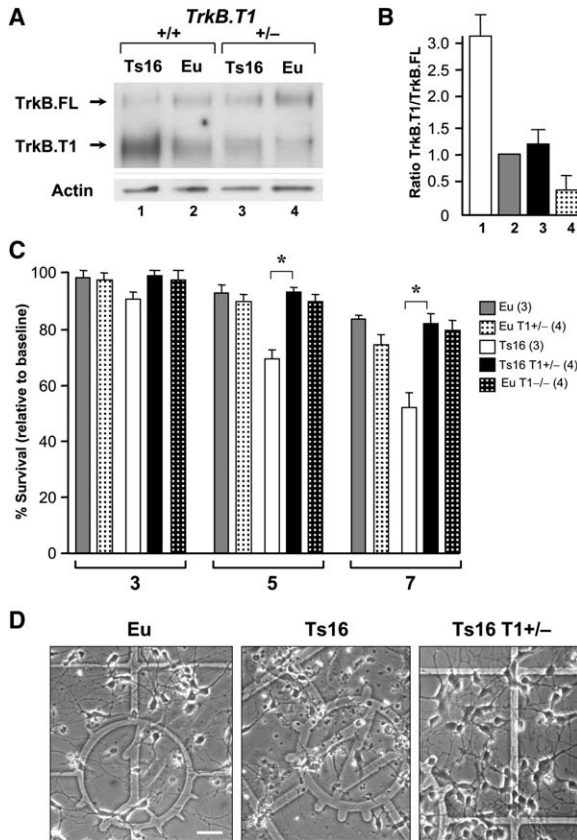


Figure 2. Reduction of TrkB.T1 Levels in the *Ts16* Mouse Restores Hippocampal Neuron Survival

(A) Deletion of one *TrkB.T1* allele restores the physiological level of TrkB.T1 in *Ts16* hippocampal neurons. Neuronal cultures were prepared from E16.5 euploid and *Ts16* littermates that were either wild-type for *TrkB.T1* or heterozygous for *TrkB.T1*. Lysates from neuronal culture from different genotypes were prepared and analyzed by Western blot using the antibody TrkB_{out}. The autoradiographs shown are representative of three independent experiments. (B) Deletion of one *TrkB.T1* allele restores the ratio of TrkB.T1/TrkB full-length (FL) in *Ts16* neurons. Blots were quantified by scanning autoradiographs into NIH Image to determine the optical density of each band. The ratio of the optical density of the TrkB.T1 and TrkB.FL bands were normalized relative to the intensity of the actin band, used as loading control, and the ratio obtained from euploid control neurons was established to be 1 (lane 2). Values represent the mean \pm SEM from three independent sets of neuronal cultures from each genotype. Lane numbers at the bottom are the same as in (A). (C) Cultured hippocampal neurons prepared from E16.5 embryos of the indicated genotypes were assayed for survival over 7 days in vitro as described in Experimental Procedures. Approximately 400 neurons for each genotype were analyzed for survival, expressed as a percentage of the number of cells at day 2 in vitro. Error bars show SEM, and the number of embryos analyzed for each genotype is indicated in parenthesis. (D) Phase-contrast images of euploid (Eu), *Ts16*, and *Ts16;TrkB.T1^{+/-}* (*Ts16T1^{+/-}*) cultured hippocampal neurons at 6 DIV. Note the extensive fragmented processes and cellular debris in the *Ts16* but not in the *Ts16;TrkB.T1^{+/-}* cultures. Scale bars, 20 μ m.

indistinguishable from wild-type (Figures 2A and 2B, lanes 2 and 3). Next, we assayed the survival of the *Ts16;TrkB.T1^{+/-}* hippocampal neurons in vitro. We cultured hippocampal neurons on gridded glass coverslips and followed the survival of individual neurons over 7 days in vitro. Consistent with our previous reports (Bambrick

et al., 1995; Dorsey et al., 2002), about 15% of wild-type, euploid neurons and 50% of *Ts16* neurons died within 7 days (Figure 2C). In contrast, the survival of *Ts16* neurons heterozygous for *TrkB.T1* was indistinguishable from wild-type euploid neuron survival, indicating a complete rescue of the premature neuronal death phenotype (Figure 2C). Interestingly, neurons lacking TrkB.T1 (Figure 2C, Eu *T1^{-/-}*) had a survival rate similar to that of control neurons, suggesting that TrkB.T1 per se is not required for neuron survival.

At day 6 in vitro, in contrast to *Ts16;TrkB.T1^{+/-}* neuron cultures, which exhibited widespread neurite fragmentation and cellular debris, neuron cultures from *Ts16;TrkB.T1^{+/-}* fetuses had complex, nonfragmented neurites and little or no cellular debris, similar to wild-type euploid neurons (Figure 2D). These findings suggest that, despite the triplication and probably dysregulation of more than 700 genes on mouse chromosome 16 (Mao et al., 2003), restoring physiological expression levels of a single gene isoform, TrkB.T1, is sufficient to rescue the premature hippocampal neuronal cell death phenotype of *Ts16* neurons.

Full-Length TrkB Response to BDNF Is Restored in *Ts16* Neurons by In Vivo Reduction of TrkB.T1

To examine the mechanism underlying the restoration of *Ts16* hippocampal neuron survival, we analyzed the BDNF-induced phosphorylation of full-length TrkB. We have previously reported that TrkB phosphorylation is decreased by ~60% in *Ts16* neurons compared with levels in euploid neurons, suggesting that increased truncated TrkB inhibits TrkB signaling (Figure 3A; Dorsey et al., 2002). However, when we reduced the levels of TrkB.T1 in vivo by deleting one copy of *TrkB.T1*, the BDNF-induced phosphorylation of full-length TrkB in neurons from *Ts16* embryos was restored to 85%–90% of wild-type euploid levels (Figures 3A and 3B). We next investigated the phosphorylation level of Akt and Erk, two important downstream proteins of TrkB-activated signaling cascades (Huang and Reichardt, 2003; Kaplan and Miller, 2000; Figures 3A and 3B). Surprisingly, we found that after treating *Ts16* neurons with BDNF, only Akt phosphorylation was significantly reduced to about 70%–75% of that of euploid neurons, while Erk phosphorylation was not affected by the triplication of chromosome 16. This intriguing result suggests that TrkB.T1 does not act in a classic dominant-negative way by reducing the general activation of the TrkB kinase receptor. Instead, it affects only specific downstream signaling pathways. Moreover, reduction of truncated TrkB.T1 to wild-type levels in *Ts16* neurons (Figures 3A and 3B; *Ts16T1^{+/-}*) normalizes Akt phosphorylation levels in response to BDNF, suggesting that the overexpression of this truncated isoform is indeed responsible for the dysregulation of Akt activation. To investigate whether TrkB.T1 differentially affects signaling to ERK and Akt, we performed a dose-response analysis of the TrkB kinase-activated signaling pathways in neurons lacking TrkB.T1. Neurons from control and *TrkB.T1* mutant embryos were isolated, cultured for 3 days, starved for 4 hr, and treated with BDNF at concentrations ranging from 1 to 100 ng/ml. Phosphorylation of the TrkB kinase receptor, as well as Erk and Akt, was increased in neurons lacking the TrkB.T1

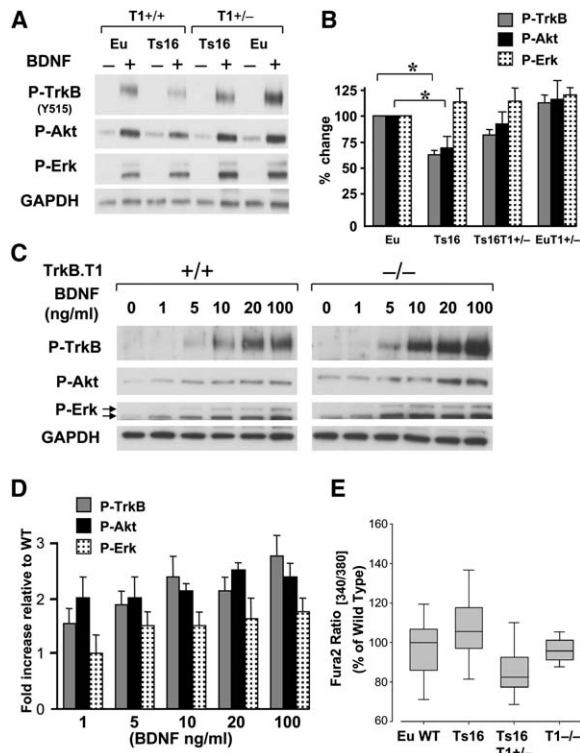


Figure 3. Reduction of TrkB.T1 in *Ts16* Neurons Restores BDNF-Induced TrkB Activation and Resting Cytoplasmic Ca^{2+} Levels
 (A) E16.5 hippocampal neuron cultures of the indicated genotypes were cultured with B27 serum supplement. On the third day in vitro, the supplement was removed for 4 hr, and neurons were either left untreated as controls or treated for 5 min with 100 ng/ml of BDNF. Lysates were harvested and analyzed by Western blot using an antibody specific for Trk phosphorylation at the Y515 site (Shc binding site) or antibodies recognizing the phosphorylated forms of Akt and Erk. The autoradiographs shown are representative of three independent sets. (B) Quantification of TrkB, Erk, and Akt activation in neurons' lysates is calculated as the ratio of band intensity value of phospho-protein and GAPDH where the ratio for euploid control neurons is set at 100%. Error bars indicate SEM ($n = 4$). * $p < 0.01$ by t test. (C) BDNF dose-response analysis on E16.5 hippocampal neuron cultures from control (+/+) and *TrkB.T1*^{-/-} neurons. Neuronal cultures were prepared and treated with BDNF concentrations ranging from 0 to 100 ng/ml as described in (A). Note the robust increase in the phosphorylation levels of both TrkB kinase and Akt in *TrkB.T1*-deficient neurons compared to the control cultures. (D) Quantification of phospho TrkB, Erk, and Akt in neurons' lysates is calculated as the ratio of band intensity value of phospho-protein and GAPDH where the ratio for euploid control neurons is set at 1. Error bars indicate SEM ($n = 3$). (E) Cytoplasmic Ca^{2+} is reduced in hippocampal neurons from *Ts16;TrkB.T1*^{+/-} mice. Box plots indicate the 25th, 50th (median), and 75th percentiles of the distribution of Fura 2 fluorescence emission ratios from euploid (Eu wt, $n = 54$), *Ts16;TrkB.T1*^{+/+} (*Ts16*, $n = 64$), *Ts16;TrkB.T1*^{+/-} (*Ts16 T1*^{+/-}; $n = 47$), and *TrkB.T1*^{-/-} (*T1*^{-/-}; $n = 87$) neurons obtained from four animals of the indicated genotypes ($N = 4$ experiments). Whisker lines indicate the range from 1% to 99% of the distribution. The *Ts16* group is significantly different from all other groups at $p < 0.05$.

isoform compared to that of control neurons (Figures 3C and 3D). However, as observed in Figure 3A, signaling to Akt appeared to be differentially affected by the loss of TrkB.T1, since Erk activation was less influenced by TrkB.T1 deficiency. This suggests that TrkB.T1 selectively modulates signaling to different downstream

pathways (Figures 3C and 3D). Notably, the basal level of Akt phosphorylation was substantially higher in TrkB.T1-deficient neurons compared to that observed in controls, suggesting that TrkB.T1 may serve as a "brake" to prevent unwanted TrkB signaling.

Taken together, these data support the notion that the overexpression of TrkB.T1 in *Ts16* neurons affects full-length TrkB-activated signal transduction but, surprisingly, this inhibition is selective to specific downstream signaling molecules. In this respect, it should be noted that while Erks have been implicated in neuronal survival and proliferation, Akt phosphorylates several proteins involved in controlling cell survival (Datta et al., 1999; Yuan and Yankner, 2000). Thus, changes in Akt phosphorylation levels may be in part responsible for the premature neuronal cell death observed in *Ts16* neurons.

Intracellular Cytoplasmic Ca^{2+} Levels Are Restored in *Ts16;TrkB.T1*^{+/-} Neurons

Ts16 neurons and astrocytes have increased levels of resting intracellular Ca^{2+} (Bambrick et al., 1997; Muller et al., 1997; Schuchmann et al., 1998). Recently, it was shown that BDNF induces an increase in cytoplasmic Ca^{2+} in astroglia. Because cultured glial cells do not express measurable levels of full-length TrkB, this response is mediated by the truncated TrkB.T1 receptor (Rose et al., 2003). However, in neurons, a physiological link between the TrkB.T1 and Ca^{2+} levels is unknown. Therefore, we investigated whether restoring normal TrkB.T1 expression would reduce resting Ca^{2+} levels in *Ts16* neurons. In agreement with previous reports, we found a small, but significant, increase in resting intracellular Ca^{2+} levels in *Ts16* hippocampal neurons ($Q = 2.424$, $p < 0.05$) between 4 and 7 days in vitro (Figure 3E). Interestingly, when TrkB.T1 expression in *Ts16* mice was reduced to euploid levels, we found a significant decrease ($Q = 5.484$, $p < 0.05$) in intracellular resting levels of cytoplasmic Ca^{2+} (Figure 3E). These data suggest that the altered Ca^{2+} homeostasis observed in *Ts16* hippocampal neurons is a consequence of TrkB.T1 dysregulation. Interestingly, complete loss of TrkB.T1 does not affect resting Ca^{2+} levels in neurons, suggesting that other mechanisms are involved in the normal control of this crucial physiological process (Figure 3E).

Ts16;TrkB.T1^{+/-} Mouse Brains Have Normal Levels of Apoptosis

Since correcting truncated TrkB.T1 levels rescues hippocampal neuron accelerated cell death, we next investigated the physiological relevance of this finding in vivo. Previous studies have shown that *Ts16* embryos have increased apoptosis in both proliferative and postmitotic zones of the developing neocortical wall (Haydar et al., 2000). Thus, we investigated whether restoration of physiological levels of TrkB.T1 receptor could rescue the increased cell death in the cortex of *Ts16* mice. For our analysis, we used two markers of apoptosis: terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining and immunocytochemical detection of cleaved (activated) caspase-3 (Figures 4A and 4B). These methods detect different stages of the apoptosis cascade and therefore provide reliable quantitative and qualitative information of cell death. First, we studied cell death occurrence within

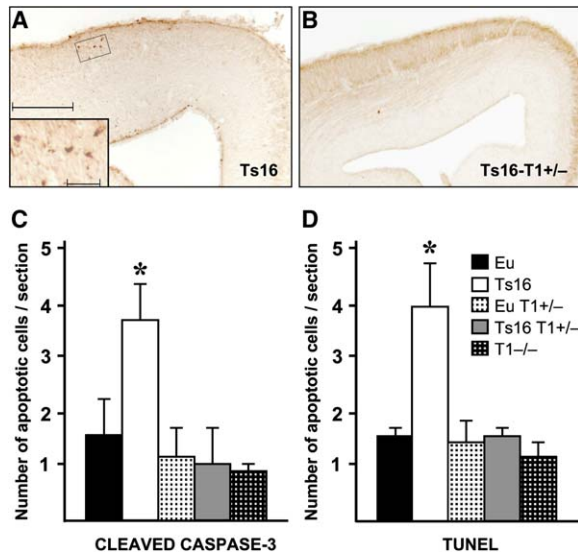


Figure 4. *Ts16* Embryos with Reduced *TrkB.T1* Levels Have Normal Cortical Apoptosis Incidence

(A and B) Light microscopy images of 20 μm sections from E16.5 *Ts16* embryos that were either wild-type (A) or heterozygous for *TrkB.T1* (B). The tissue sections were processed immunohistochemically with an antibody directed at cleaved caspase-3. Images in (A) and (B) are at 100 \times ; scale bar, 100 μm . Inset in (A) is at 200 \times magnification; scale bar, 25 μm . (C and D) Dying cells throughout the cortical wall of embryos at day 16.5 of development were quantified by counting cells positive for cleaved caspase-3 (C) or by the TUNEL method (D) as described in [Experimental Procedures](#). Note that *Ts16* embryos have a 3- to 4-fold increase in the number of apoptotic cells as previously reported ([Haydar et al., 2000](#)). Deletion of one copy of *TrkB.T1* restores the number of apoptotic cells to normal levels. Data are from two independent sets of animals, one set for cleaved caspase-3 ($n = 4$ for each genotype) and one set for the TUNEL analysis ($n = 4$ for each genotype). Mean \pm SEM. Data were analyzed by Student's *t* test. * $p < 0.05$ relative to Eu control embryos.

the cortex of euploid and *Ts16* embryos and found that, as previously reported, they contained an average of 1.5 TUNEL and cleaved caspase-3 positive neurons per section while the *Ts16* embryos had about four TUNEL and cleaved caspase-3 positive neurons per section (Figure 4) ([Haydar et al., 2000](#)). In contrast to *Ts16* brains, the average number of TUNEL and cleaved caspase-3 positive neurons per section of the *Ts16;TrkB.T1*^{+/-} brains was 1.0 to 1.5, indistinguishable from wild-type Eu cortices and significantly different from that of *Ts16* embryos (Figures 4C and 4D). Taken together, these results suggest that reducing the expression of *TrkB.T1* to physiological levels normalizes the incidence of neuronal cell death in the brains of *Ts16* embryos. It should be noted that, consistent with what has been observed in isolated hippocampal neurons, complete loss of the *TrkB.T1* receptor does not increase the number of apoptotic neurons in the embryonic cortex, suggesting that, also in vivo, *TrkB.T1* does not affect directly the cellular processes that trigger neuronal cell death (Figures 4C and 4D).

Discussion

In this study, we demonstrate that correcting truncated *TrkB.T1* receptor expression to physiological levels res-

cues the accelerated death of *Ts16* hippocampal neurons and normalizes the level of apoptosis in the cortex of *Ts16* fetuses. In turn, rescued neurons show both an increase in the phosphorylation level of the *TrkB* tyrosine kinase receptor and Akt in response to BDNF and a restoration of intracellular Ca^{2+} levels.

Alterations in neurotrophins or their Trk receptor levels have been reported in a variety of neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and Alzheimer's, Huntington's, and Parkinson's diseases (reviewed in [Dawbarn and Allen, 2003](#)). However, it is still unclear whether changes in expression of these receptors are involved in the pathogenic process or are an indirect effect of the disease. A major problem with testing this hypothesis has been the limited number of suitable models. *Ts16* hippocampal neurons provide a tool to study the genetic abnormalities underlying neuronal cell death. The cause of the accelerated cell death has the potential to be multigenic, since hundreds of genes are dysregulated in trisomies ([Mao et al., 2003](#)). Surprisingly, we found that an alteration in *TrkB* receptor signaling is sufficient for the development of this phenotype, suggesting that dysregulation of a single gene is sufficient to cause cellular alterations resulting in neuron death. It should be noted that since the *TrkB* gene is located on mouse chromosome 13 and consequently not triplicated in *Ts16*, the altered expression of *TrkB* isoforms observed in *Ts16* neurons must be secondary to the increased gene dosage of one or more chromosome 16 genes.

Expression of *TrkB* receptor isoforms is dynamically, yet tightly, regulated during development of the mammalian or chicken nervous system, suggesting a critical role for this receptor. Expression of the full-length *TrkB* precedes that of the truncated isoforms ([Allendoerfer et al., 1994; Escandón et al., 1994](#)). Interestingly, in the mammalian cortex, the shift to a predominance of truncated *TrkB* occurs at times that correlate with the onset of cell death and maturation of axonal connections ([Allendoerfer et al., 1994; Ohira et al., 1999](#)). Thus, imbalances in *TrkB* receptor isoform levels in pathological conditions may lead to cell death with a mechanism similar to that used during the critical period of cell death and axonal remodeling associated with the nervous system developmental plasticity.

Interestingly, we found that *TrkB.T1* overexpression in *Ts16* neurons has a selective effect on Akt activation, a downstream signaling molecule associated with cell survival, while BDNF stimulation of ERK phosphorylation is not affected in *Ts16* neurons ([Kaplan and Miller, 2000](#)). This is a surprising result because it suggests that truncated *TrkB* does not affect *TrkB* signaling by a simple dominant-negative mechanism of the full-length receptor. Instead it suggests that truncated *TrkB* may affect, in a discrete way, the complex balance of interactions between the activated full-length *TrkB* receptor and the numerous cytoplasmic adaptor proteins involved in transducing its signals ([Huang and Reichardt, 2003; Kaplan and Miller, 2000; Segal, 2003](#)). In support of this hypothesis is also the finding that, when *TrkB.T1* is completely ablated in neurons, we still observed a differential effect of signaling to Akt and Erk, with Akt being more robustly phosphorylated in response to BDNF (Figures 3C and 3D). Truncated

TrkB.T1 receptors are upregulated in the postnatal mammalian brain at the time when synaptic transmission and plasticity and memory formation become key processes in brain development. Interestingly, BDNF-activated Erk signaling has been heavily implicated in these brain activities (Alonso et al., 2002, 2004; Blanquet, 2000; Finkbeiner et al., 1997; Gottschalk et al., 1999; Pizzorusso et al., 2000). Thus, our findings raise the intriguing possibility that the TrkB.T1 physiological upregulation in the mammalian brain may be used to control Akt activation, leaving Erk signaling unaffected to modulate synaptic plasticity events and memory formation.

Mice lacking *TrkB* or its ligand *BDNF* display a significant increase of apoptosis in different areas of the developing CNS, including the hippocampus, cortex, and cerebellum (Alcántara et al., 1997; Minichiello and Klein, 1996; Schwartz et al., 1997). These losses are not severe, most likely because of the presence of multiple pathways promoting the survival of CNS neurons or the compensatory effect of the TrkC receptors that are frequently coexpressed in neurons of the CNS (Alcántara et al., 1997; Minichiello and Klein, 1996; Miranda et al., 1993). However, these data are in agreement with the mild, yet significant, increase of neuronal cell death observed in the cortex of the *Ts16* embryos. In addition, this phenotype can be exacerbated by the in vitro culture conditions that can block some of the compensatory mechanisms present in vivo. Thus, our data support the notion that an intact BDNF/TrkB signaling system is required for the maintenance of CNS neurons and establish TrkB.T1 as a critical player in the tight control of this important ligand/receptor system.

We previously reported a selective failure of BDNF to support the survival of *Ts16* hippocampal neurons due to the elevated expression of TrkB.T1 relative to full-length TrkB and that this defect could be corrected by overexpressing full-length TrkB (Dorsey et al., 2002). Now we report that reducing the expression of TrkB.T1 to the level found in euploid neurons can restore BDNF-induced survival of *Ts16* neurons. These data suggest that neurodegeneration may not only be the result of a diminished supply of neurotrophins and provide direct evidence that neurons must express the correct set of receptor isoforms to transduce a proper survival signal in response to neurotrophins.

Interestingly, overexpression of truncated *TrkB* has also been reported in the hippocampus of human Alzheimer's disease patients (Ferrer et al., 1999), suggesting that a similar mechanism may participate in hippocampal neuron degeneration in this disease. Moreover, in ALS patients, *BDNF* mRNA and protein are dramatically upregulated in muscle as well as total *TrkB* mRNA in the spinal cord (Kust et al., 2002; Mutoh et al., 2000). Yet, phosphorylation of the TrkB receptor is reduced (Mutoh et al., 2000). Thus, it is possible that TrkB signaling impairments in ALS are not at the level of neurotrophin supply but rather at the level of the TrkB receptor isoforms being expressed. Determination of truncated and full-length TrkB receptor ratios should help in identifying whether ALS and *Ts16* neurons have similar deficits and may help to explain why therapeutic use of BDNF has been unsuccessful in ALS clinical trials (Ochs et al., 2000).

We have found that, in *Ts16* hippocampal neurons, restoration of physiological levels of TrkB.T1 corrects resting Ca^{2+} levels. While it was known that truncated TrkB alone mediates the regulation of intracellular Ca^{2+} signaling by BDNF in astrocytes (Rose et al., 2003), the function of TrkB.T1 in neurons, which express both full-length and truncated TrkB isoforms, is more complex. Our data suggest that, in neurons, proper TrkB.T1 levels (or the relative proportion of full-length TrkB to TrkB.T1) are critical for maintaining Ca^{2+} homeostasis. Since even small increases in cytoplasmic Ca^{2+} can have deleterious consequences, including decreased neuronal survival (Johnson et al., 1992), it is possible that the correction in the intracellular Ca^{2+} level by reduction of TrkB.T1 may contribute to the rescue of the *Ts16* accelerated neuronal cell death.

Taken together, our data provide evidence that relatively small imbalances in the physiological levels of TrkB receptor isoforms affect neuronal survival by altering both BDNF-induced prosurvival signaling and Ca^{2+} homeostasis. Moreover, they suggest that the success of exogenous neurotrophin application to reduce or prevent cell death in neurodegenerative disorders may depend upon the neuron's ability to properly regulate the expression of neurotrophin receptor isoforms and, consequently, its survival.

Experimental Procedures

Generation of *TrkB.T1* Mutant Mice

The targeting vector to conditionally remove the TrkB.T1-specific coding exon was constructed by a recombinogenic cloning strategy (Liu et al., 2003) using a murine BAC clone (RP23 library, Invitrogen). An upstream loxP site, containing a SpeI (S) restriction site, was placed 300 bp 5' of the T1 coding sequence. The pGK-neomycin (NEO) resistant cassette flanked by loxP and frt sites was placed 1 kb 3' of the T1 coding sequence (Figure 1). The targeting vector was electroporated in the CJ7 embryonic stem cell line (129/sv), as described (Tessarollo, 2001), and recombinant clones obtained at a frequency of 1/44 were injected into C57BL/6 blastocysts to produce chimeras that transmitted the targeted TrkB.T1 allele to the progeny (Bonin et al., 2001). Following the NEO cassette and the TrkB.T1 exon removal, breeding of two *TrkB.T1*^{+/-} mice gave rise to homozygous mutants at a frequency of 25%.

Western Blot Analysis

Cells were lysed and analyzed by Western blot analysis as described (Dorsey et al., 2002). The rabbit polyclonal antibody directed to an extracellular epitope of TrkB (trkB_{out}) was generously provided by Dr. Deborah Morrison (NCI-Frederick). The mouse monoclonal raised to the extracellular TrkB epitope, the polyclonal antibodies specific for phospho-Trk (#9141), phospho-Akt (#9271), and phospho-MAPK/ERK1/2 (#9106) were obtained from Cell Signaling. The anti actin (#SC-1616) and GAPDH (MAB374) antibodies were, respectively, from Santa Cruz and Chemicon International. BDNF was from Upstate Biotechnology. Autoradiographs were scanned at 600 dpi using a Hewlett-Packard scanner, and quantitation of band intensity was performed using NIH Image v1.62 (National Institutes of Health). The activation of TrkB, Akt, and ERK by BDNF was quantified by calculating the ratios of band intensity of Phospho-TrkB, Erk, or AKT and band intensity of GAPDH. The values obtained from euploid controls were set as 100%.

Generation of Trisomic Mice and Karyotyping

Robertsonian mice (Jackson Laboratory, Bar Harbor, ME) were mated with wild-type or *TrkB.T1* homozygous C57BL/6J mice at generation N4 to generate wild-type and *Ts16* mice heterozygous for *TrkB.T1*. The next day was designated as day 0.5 of gestation (E 0.5). Normal and *Ts16* fetuses were easily distinguishable; however, mice were also karyotyped by fluorescence in situ

hybridization (FISH) using probes specific for chromosome(s) 6, 16, and 17 to confirm visual phenotyping.

Neuron and Astrocyte Cultures

Hippocampal neurons were dissected and cultured from euploid and *Ts16* wild-type and *TrkB.T1^{+/−}* E16.5 mice in minimal essential medium (MEM) and the serum-free supplement B27, as previously described (Bambrick et al., 1995). Neurons were plated at 10^4 cells/cm² on 12 mm glass coverslips etched with a lettered grid (Eppendorf AG, Hamburg, Germany) for survival experiments and at 5×10^5 cells/35 mm dish for Western blots. Glass coverslips were coated with poly-L-lysine (Sigma, St. Louis, MO) and mouse laminin. Plastic dishes were coated with poly-L-lysine alone. The purity of hippocampal neuron cultures was established by immunostaining for glial fibrillary acidic protein (GFAP), which revealed no differences in the proportion of astrocytes (<5%) in euploid and *Ts16* cultures (data not shown). Unless otherwise indicated, all cell culture reagents were obtained from Invitrogen (Carlsbad, CA).

Astrocytes cultures were obtained from E16.5 embryos as described previously (Bambrick et al., 1997). Glial fibrillary acidic protein (GFAP) immunostaining was conducted to verify that the majority (>90%) of cells were astrocytes (data not shown).

Measurement of Neuron Survival

At 2 days in vitro (DIV), all live neurons in each of four randomly selected $175 \times 175 \mu\text{m}$ fields per coverslip (identified by the etched grid) and at least eight coverslips per genotype ($n = 400+$) were counted using phase-contrast microscopy (Dorsey et al., 2002). Neurons were counted every 24 hr, and survival was expressed as a percentage of cells present at 2 DIV that remained at 7 DIV. The significance of the difference between genotypes was determined by Student's *t* test.

Dye Loading and Fluorescence Measurement

Isolated primary hippocampal neurons were loaded with Fura 2AM (3 μM ; Molecular Probes, Inc., OR) in Physiological Saline Solution (PSS; NaCl 140 mM, KCl 4.0 mM, CaCl₂ 1.8 mM, KH₂PO₄ 1.0 mM, MgCl 1.4 mM, HEPES 10.0 mM, glucose 11.5 mM) for 40 min, washed twice (10 min) with PSS, and rested for 30 min. Fields of neurons were then imaged (Olym. IX-50; $40\times$ H20 Obj.) with alternating excitation (340 nm, 380 nm; Sutter DG-4; Chroma filter set) while the emission at 515 nm was collected (Roper CoolSnap HQ) as described previously (Chun et al., 2003). On archived image sequences (five samples/field; 0.2 Hz) emission ratios (340ex/380ex) were calculated for each neuron following background correction (IPlab 4.1) with the mean ratio used as the value for relative $[\text{Ca}^{2+}]_{\text{free}}$. Results are from *Eu^{+/+}* ($n = 54$), *Ts16^{+/+}* ($n = 64$), *Ts16;TrkB.T1^{+/−}* ($n = 47$), and *TrkB.T1^{−/−}* ($n = 87$) neurons obtained from at least four animals per genotype ($N = 4$ experiments). Relative $[\text{Ca}^{2+}]_{\text{free}}$ between groups was compared using a nonparametric, Kruskal-Wallis analysis of variance on ranks followed by a Dunns post hoc analysis (Sigma Stat 3.0; SPSS Inc.). Significance between groups was evaluated at a level of $p < 0.05$.

Detection and Quantitation of Apoptotic Cells

E16.5 fetal brains were collected, fixed in 4%PFA, and cryoprotected in 30% sucrose for cryostat sectioning. Twenty-micron coronal sections taken at approximately 200 μm caudal to the interventricular foramen, where the hippocampus appears (Jacobowitz and Abbott, 1998; plates 3–5), were analyzed for cell death in the cortical wall (Haydar et al., 2000). Sections to be analyzed for cleaved caspase-3 (1:100; Cell Signaling) immunohistochemistry analysis, or apoptosis analysis were processed by the avidin-biotin method (ABC kits, Vector Labs) or with the ApopTag Peroxidase *InSitu* Oligo Ligation Apoptosis detection Kit (Chemicon), respectively. Every third section was analyzed. Cells were counted from the lateral ventricle to the cortical surface in a 100 μm square located at a consistent position on the dorsal pallium, midway between the medial and lateral borders of the lateral ventricle following a 2D counting method (Haydar et al., 1996, 2000). The number of positively stained cells was expressed as the mean \pm SEM per animal and was used as the unit of data analysis.

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