Knocking the NT4 gene into the BDNF locus rescues BDNF deficient mice and reveals distinct NT4 and BDNF activities

Guoping Fan[1], Christophe Egles[2,3], Yi Sun[4], Liliana Minichiello[5], John J. Renger[2,3], Rüdiger Klein[5], Guosong Liu[2,3] and Rudolf Jaenisch[1,2]

[1] *Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, Massachusetts 02142, USA*

[2] *Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, Massachusetts 02139, USA*

[3] *Center for Learning and Memory and Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA*

[4] *Division of Neuroscience, Children's Hospital and Department of Neurobiology, Harvard Medical School, 300 Longwood Avenue, Boston, Massachusetts 02115, USA*

[5] *European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69117 Heidelberg, Germany*

Correspondence should be addressed to R.J. (jaenisch@wi.mit.edu)

To directly compare biological activities of the neurotrophins NT4 and BDNF *in vivo*, we replaced the BDNF coding sequence with the NT4 sequence in mice (*Bdnf*$^{nt4\text{-}ki}$). Mice expressing NT4 in place of BDNF were viable, in contrast with BDNF null mutants, which die shortly after birth. Although the *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ and wild-type *Bdnf*$^{+/+}$ alleles yielded similar levels of NT4 and BDNF proteins, NT4 supported more sensory neurons than BDNF and promoted functional synapse formation in cultured hippocampal neurons. Homozygous *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ mice showed reduced body weight, infertility and skin lesions, suggesting unique biological activities of NT4 *in vivo*. The distinct activities of NT4 and BDNF may result partly from differential activation of the TrkB receptor and its down-stream signals.

Neurotrophins are a family of growth factors regulating the survival, differentiation and mature function of a variety of peripheral and central neurons[1,2]. In mammals, this family includes nerve growth factor (NGF)[3], brain-derived neurotrophic factor (BDNF)[4], neurotrophin-3 (NT3)[5–9] and neurotrophin-4/5 (NT4)[10,11]. The diverse functions of neurotrophins are mediated by their specific interactions with tyrosine kinase Trk receptors and a common p75 neurotrophic receptor (p75^{NTR})[12,13]. These neurotrophins and their Trk receptors have distinct specificities: NGF signals through the TrkA receptor, both BDNF and NT4 bind to the TrkB receptor, and NT3 interacts primarily with TrkC and, to a lesser extent, with TrkA and TrkB[12]. These specificities are confirmed *in vivo* after analysis of mutant mice lacking either neurotrophins or corresponding Trk receptors, which exhibit largely overlapping neuronal deficits[14].

Although both NT4 and BDNF bind to the TrkB and p75^{NTR} receptors with equal affinity[15–17] and exhibit similar biological activities in many *in vitro* assays[18,19], NT4 and BDNF mutant mice exhibit quite contrasting phenotypes. Whereas NT4$^{-/-}$ mice are viable and fertile with only a mild sensory deficit[20,21], BDNF$^{-/-}$ mice die during early postnatal stages with severe neuronal deficits and behavioral symptoms[22,23]. The different phenotypes of BDNF and NT4 mutants suggest that endogenous NT4 and BDNF have diversified their *in vivo* functions. The difference between BDNF and NT4 mutants could be due to different expression patterns of the BDNF and NT4 gene, or alternatively, to unique biological activities of BDNF and NT4 proteins *in vivo*. To distinguish between these two possibilities, we took the approach of knocking NT4 into the BDNF gene locus to examine whether NT4 can substitute for BDNF functions. We further contrasted overlapping and distinct functions of NT4 and BDNF *in vivo* and investigated whether a divergence of TrkB signaling could be partly responsible for distinct activities of NT4 and BDNF.

Results

A targeting vector was constructed which introduced in frame all NT4 coding sequences after the first ATG of the BDNF coding sequences at the fifth exon of the *Bdnf* gene (**Fig. 1a**). BDNF proteins cannot be produced from this modified gene locus since a portion of the BDNF coding region was deleted as in a previous BDNF null mutation[22]. The Kozak consensus sequence of the *Bdnf* gene was preserved for the inserted NT4 cDNA in order to achieve the same translational initiation efficiency as the endogenous BDNF mRNA. A positive/negative selection cassette (CMV-hygro/tk) flanked by two loxP sites was inserted right behind the stop codons of the inserted NT4 (**Fig. 1a**). This targeting vector was transfected into J1 embryonic stem (ES) cells, and homologously recombined ES clones were identified with southern blots (**Fig. 1b**). Targeted ES cell clones were transiently transfected with the Cre recombinase expression plasmid pMC-Cre to delete the loxP-flanked selection cassette (**Fig. 1a** and **c**). The resulting new *Bdnf* allele, referred to here as *Bdnf*$^{nt4\text{-}ki}$, expressed the NT4 transgene under the control of the endogenous *Bdnf* promoter.

Two independent ES clones carrying the *Bdnf*$^{nt4\text{-}ki}$ allele were used to produce transgenic mice by the standard blastocyst injection method. The *Bdnf*$^{+/nt4\text{-}ki}$ heterozygous mice from both ES lines were normal and healthy, and were crossed with the previously described *Bdnf*$^{+/-}$ animals[22] to produce *Bdnf*$^{nt4\text{-}ki/-}$ mice which expressed one copy of the *Bdnf*$^{nt4\text{-}ki}$ allele under the con-

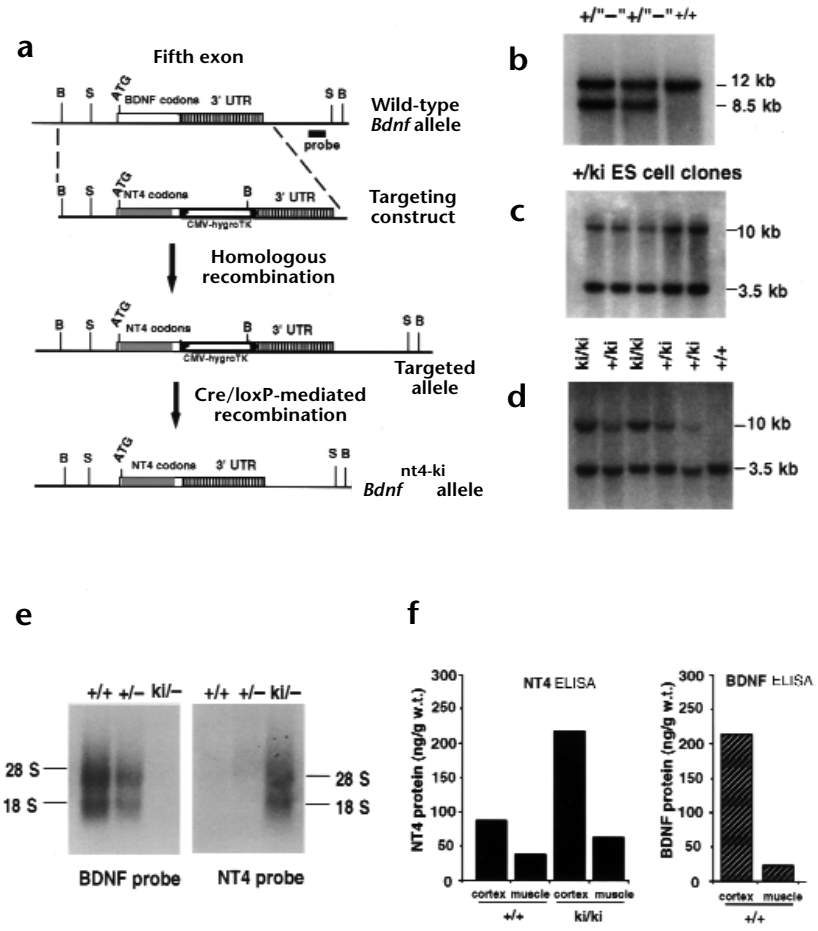

Fig. 1. Replacement of the BDNF coding sequence with NT4 codons through the Cre-loxP system. (**a**, top) Diagram of the *Bdnf* gene locus. The box represents the fifth exon of the *Bdnf* gene, which contains all the BDNF codons starting with the first ATG shown at the left end. The hatched area represents 3′ UTR for BDNF, which contains two different polyA sites. (**a**, second line) Targeting construct in which the shaded area represents NT4 codons. Black triangles represent two loxP sites which flank a selection cassette of CMV-hygroTK. (**a**, third line) Gene structure after gene targeting. (**a**, fourth line) Structure of the final $Bdnf^{nt4\text{-}ki}$ allele after cre-loxP-mediated recombination. B, BglII; E, EcoRI; S, SacI. (**b**) Southern blot of targeted ES cell clones. DNA samples were digested with BglII, and then analyzed with the external probe shown in (**a**); +/+, wild-type clones (12 kb); +/"–", targeted clones (8.5 kb). (**c**) Southern analysis of $Bdnf^{nt4\text{-}ki}$ ES cell clones. DNA samples were digested with SacI and probed with NT4 cDNA, which hybridized to the 10-kb $Bdnf^{nt4\text{-}ki}$ allele, and 2 copies of the endogenous *Nt4* gene (3.5 kb). (**d**) Southern analysis of offspring from intercrossing $Bdnf^{nt4\text{-}ki}$ heterozygous mice. Tail DNAs were digested with SacI and probed with NT4 cDNA as in (**c**). +/+, wild type; +/ki, heterozygous; ki/ki, homozygous $Bdnf^{nt4\text{-}ki/nt4\text{-}ki}$. Compare the intensity of the $Bdnf^{nt4\text{-}ki}$ allele with the endogenous *Nt4* gene for determining homozygous and heterozygous genotypes. (**e**) Northern blots of the endogenous BDNF and knock-in NT4 mRNAs. RNA blots containing 40 μg of total RNA from adult cortices in each lane were hybridized with either a 500-bp BDNF probe containing the BDNF coding sequences or a 750-bp NT4 cDNA probe; +/+, wild type; +/–, $Bdnf^{+/-}$ mutant; ki/–, $Bdnf^{nt4\text{-}ki/-}$ mutant. Note that the endogenous NT4 transcript is below the threshold of detection of total RNA Northern blot analysis. The chimeric mRNA transcripts containing NT4 codons and 5′ and 3′ BDNF UTRs are readily detected at the expected sizes and comparable levels compared to the endogenous BDNF transcripts. (**f**) Elisa analysis of NT4 and BDNF proteins in the cortex and muscle samples from wild-type (+/+) and $Bdnf^{nt4\text{-}ki/nt\text{-}ki}$ (ki/ki) mice. Samples from +/+ and ki/ki as well as $Nt4^{-/-}$ and $Bdnf^{-/-}$ control samples were assayed in duplicate against the standard curves of purified recombinant NT4 and BDNF proteins as described in Methods. $Nt4^{-/-}$ and $Bdnf^{-/-}$ samples had a background value in NT4 and BDNF ELISA assays, respectively, which was subtracted from the raw readings of +/+ and ki/ki samples to generate the final values in the figure. In BDNF ELISA assays, $Bdnf^{nt4\text{-}ki/nt\text{-}ki}$ samples yielded similar background values to $Bdnf^{-/-}$ samples, and thus were omitted for the simplicity of the figure. Numbers in the figures represent averaged values from two independent experiments of ELISA analysis on two sets of adult wild-type and $Bdnf^{nt4\text{-}ki/nt\text{-}ki}$ mice.

trol of the BDNF promoter, but were deficient in BDNF. In contrast with the early postnatal death of $Bdnf^{-/-}$ mutants, $Bdnf^{nt4\text{-}ki/-}$ mice were phenotypically indistinguishable from their wild-type ($Bdnf^{+/+}$), $Bdnf^{+/nt4\text{-}ki}$ and $Bdnf^{+/-}$ littermates, indicating that one copy of the $Bdnf^{nt4\text{-}ki}$ allele was sufficient to support the survival of BDNF-deficient mice. To verify that $Bdnf^{nt4\text{-}ki/-}$ animals indeed expressed NT4 instead of BDNF from the *Bdnf* gene locus, we examined levels of endogenous BDNF and knock-in NT4 mRNAs in wild-type, $Bdnf^{+/-}$ and $Bdnf^{nt4\text{-}ki/-}$ mice by a Northern blot. Transcripts containing NT4 codons and BDNF 5′ and 3′ UTRs were readily detected in the $Bdnf^{nt4\text{-}ki/-}$ brain sample at the expected sizes and levels by a NT4 cDNA probe, but not by a probe containing the BDNF coding region. Adult $Bdnf^{nt4\text{-}ki/-}$ mice were fertile and showed normal behavioral phenotypes. The observation that knock-in NT4 rescued BDNF-deficient mice confirms that functions of NT4 and BDNF overlap *in vivo*.

Heterozygous $Bdnf^{+/nt4\text{-}ki}$ mice were crossed to produce homozygous $Bdnf^{nt4\text{-}ki/nt4\text{-}ki}$ animals, which were also viable in adulthood. ELISA analysis of NT4 and BDNF proteins in cortex and muscle extracts confirmed that appropriate levels of NT4 protein, instead of BDNF protein, were present in adult $Bdnf^{nt4\text{-}ki/nt4\text{-}ki}$ animals (**Fig. 1f**). However, $Bdnf^{nt4\text{-}ki/nt4\text{-}ki}$ mice were markedly smaller than wild-type or heterozygous littermates from the newborn stage. At 2–6 months of age, homozygous $Bdnf^{nt4\text{-}ki/nt4\text{-}ki}$ mice weighed 30% less than littermate wild-type controls (wild type, 26.0 ± 1.6 g, $n = 6$; ki/ki, 18.2 ± 1.6 g, $n = 5$; $p < 0.01$, *t*-test). These adult homozygous $Bdnf^{nt4\text{-}ki/nt4\text{-}ki}$ mice frequently scratched their ears and neck regions and developed cranial and neck skin lesions, suggesting that the cutaneous sensory function in $Bdnf^{nt4\text{-}ki/nt4\text{-}ki}$ mutants may have been altered. Most $Bdnf^{nt4\text{-}ki/nt4\text{-}ki}$ mice were infertile; a small fraction of homozygous males were subfertile and sired only a few litters in young adulthood. These results argue that the biological activities of NT4 and BDNF, though similar, are not identical *in vivo*. The behavioral phenotypes observed in $Bdnf^{nt4\text{-}ki/nt4\text{-}ki}$ mice are probably attributable to the presence of excess NT4 biological activity, but not the absence of BDNF; adult $Bdnf^{nt4\text{-}ki/-}$ animals producing no BDNF only occasionally showed scratching behavior or skin lesions.

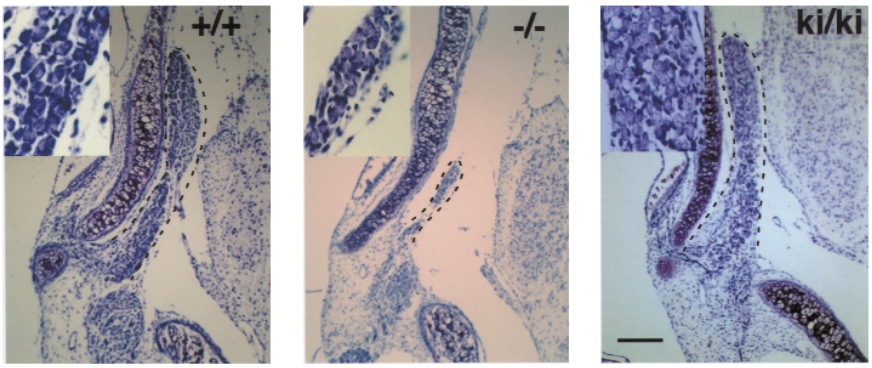

Fig. 2. Rescue of vestibular neurons in *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ newborn mice. Coronal sections of newborn heads from wild-type (+/+), *Bdnf*$^{-/-}$ (–/–) and *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ (ki/ki) mice were stained with cresyl violet to visualize sensory ganglia. The vestibular sensory ganglia were outlined in the pictures, which represented the maximal sizes seen in the serial sections. The inlets in each panel showed a portion of vestibular neurons at a high magnification for each genotype. Vestibular ganglia from wild-type and *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ mice are obviously larger than those from *Bdnf*$^{-/-}$ mice. Scale bar, 135 μm.

To distinguish in detail the distinct but overlapping biological activities of NT4 and BDNF *in vivo*, we examined whether knock-in NT4 would rescue many neural defects caused by BDNF deficiency. In the peripheral nervous system, deletion of the BDNF gene leads to specific neuronal losses in cranial sensory ganglia, and endogenous NT4 does not compensate for this deletion[25–27]. We compared the total number of sensory neurons in cranial vestibular, geniculate and nodose-petrosal ganglia in wild-type and homozygous *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ mice. Numbers of neurons in *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ mice were similar to those of wild-type animals (**Table 1**), suggesting that knock-in NT4 can support BDNF-dependent sensory neurons *in vivo*. Vestibular neurons were actually significantly more abundant in *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ mice than in wild-type animals (**Table 1** and **Fig. 2**), suggesting that NT4 is a more active trophic factor than BDNF for vestibular neurons. Because *Bdnf*$^{+/-}$ mice showed a clear dosage effect on sensory neuron survival[25,27], we further compared the number of sensory neurons between *Bdnf*$^{+/-}$ and *Bdnf*$^{nt4\text{-}ki/-}$ mice. In all three sensory ganglia, one copy of the *Bdnf*$^{nt4\text{-}ki}$ allele supported 17%–27% more sensory neurons than one copy of the endogenous *Bdnf* allele (**Table 1**). This effect was not transient, but persisted in adult mice, as the number of neurons in adult *Bdnf*$^{nt4\text{-}ki/-}$ nodose-petrosal ganglia was significantly higher than that in age-matched *Bdnf*$^{+/-}$ animals (data not shown). Thus, NT4 is actually a more potent survival factor than BDNF for many types of sensory neurons that normally depend on BDNF *in vivo*.

BDNF deficient mice also show various defects in the development and function of the CNS, including impaired synaptic function in hippocampal neurons[23,28–31]. Therefore, we were interested in determining whether NT4 can substitute for BDNF function in the survival and synapse development of hippocampal neurons. Newborn *Bdnf*$^{-/-}$ hippocampal neurons survived very poorly in culture compared with wild-type cells, suggesting that neuronal survival requires autocrine or paracrine action of endogenous BDNF (mean number of neurons in 10-day old cultures ± s.e. per mm^2, *Bdnf*$^{-/-}$, 59.1 ± 3.6; *Bdnf*$^{+/+}$, 143.9 ± 17.3; $p < 0.001$). In contrast, hippocampal neurons from *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ mice survived well throughout the culture period examined. After 10 days in culture, *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ and wild-type cultures had similar numbers of neurons, suggesting that NT4 can replace endogenous BDNF in promoting survival of cultured hippocampal neurons (*Bdnf*$^{+/+}$, 166.0 ± 13.4; *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$, 182.2 ± 13.2, $p = 0.47$).

Cultured hippocampal neurons grow processes and form functional synapses within two weeks[32]. Knowing the neuronal density in the *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ culture was equivalent to wild-type levels, we assessed the role of NT4 in synaptogenesis. After ten days in culture, wild-type, *Bdnf*$^{+/nt4\text{-}ki}$ and *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ neurons showed similar densities of synapsin I spots, indicating the presence of similar number of synaptic contacts (**Fig. 3**). We stained cultures with FM1-43 dye (FM) to quantitate the number of functional synapses (defined as those with active synaptic vesicle turnover)[33]. Strikingly, the density of FM punctae on neurites in *Bdnf* $^{+/nt4\text{-}ki}$ and *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ cultures was significantly higher

Table 1. Numbers of cranial sensory neurons in wild-type and mutant mice.

	Wild type		***Bdnf*$^{+/-}$**		***Bdnf*$^{nt4\text{-}ki/-}$**		***Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$**		***Bdnf*$^{-/-}$ (data from ref. 20)**
Ganglion	Mean no. of neurons	% of control	Mean no. of neurons	% of control	Mean no. of neurons	% of control	Mean no. of neurons	% of control	% of control
Vestibular	6490 ± 213 (n = 8)	100 ± 3	4178 ± 200 (n = 4)	64 ± 3**	5918 ± 426 (n = 5)	91 ± 7#	7611 ± 408 (n = 6)	117 ± 6*	(24 ± 3)
Nodose-petrosal	6859 ± 243 (n = 6)	100 ± 4	4932 ± 546 (n = 4)	72 ± 8**	6452 ± 602 (n = 4)	94 ± 9	7283 ± 199 (n = 3)	106 ± 3	(43 ± 5)
Geniculate	1433 ± 38 (n = 6)	100 ± 3	1162 ± 84 (n = 4)	81 ± 6*	1405 ± 57 (n = 3)	98 ± 4	1475 ± 79 (n = 5)	103 ± 6	(52 ± 12)

Counts were displayed as number ± s.e. * $p < 0.05$, ** $p < 0.01$ (unpaired t-test, when compared to the wild-type control). # $p < 0.05$ (unpaired t-test, when compared to the *Bdnf*$^{+/-}$ group).

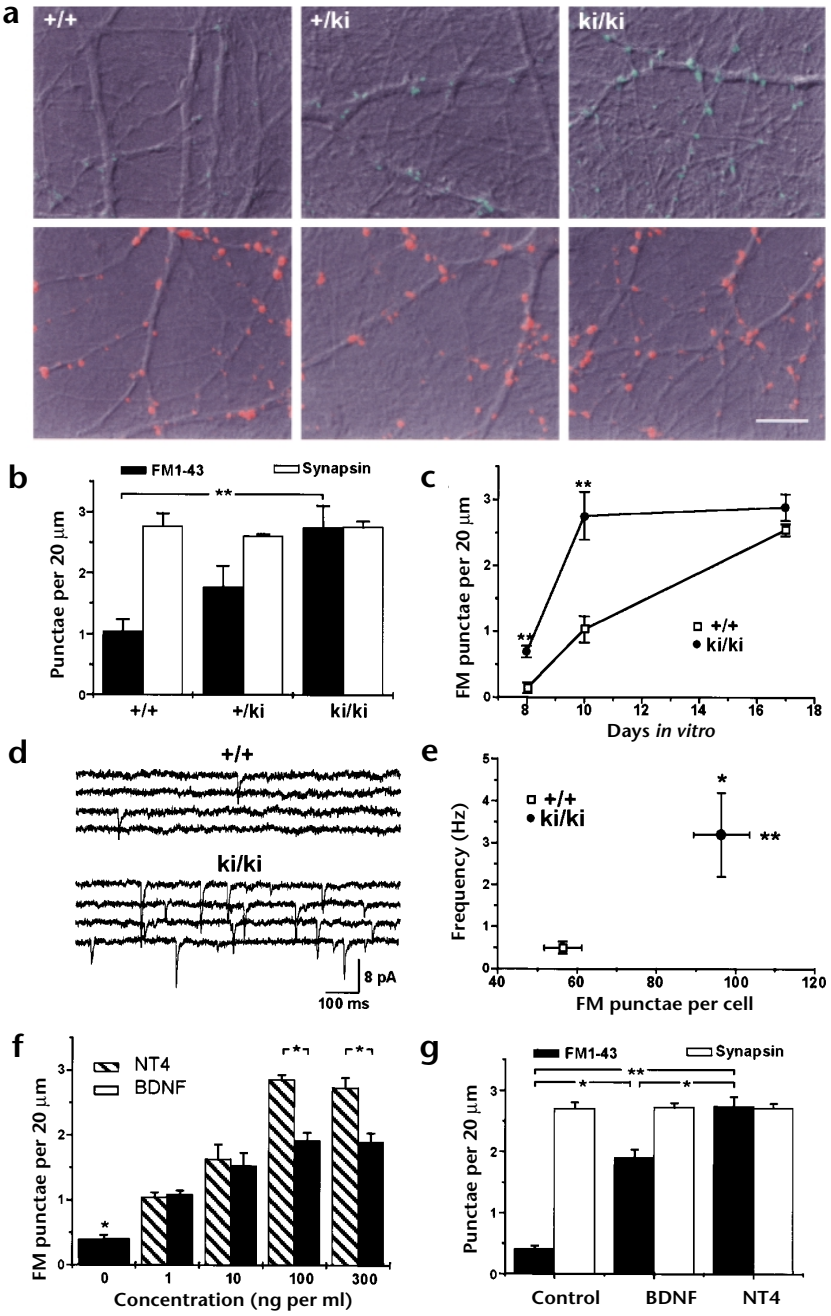

Fig. 3. Knock-in NT4 promotes functional synapse formation in cultured hippocampal neurons. (**a**) Wild type (+/+), *Bdnf*$^{+/nt4\text{-}ki}$ (+/ki) and *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ (ki/ki) hippocampal cultures at ten days were stained with FM1-43 dye (green) and anti-synapsin I antibodies (red). Scale bar, 10µm (**b**) Quantitation of the density of FM1-43 and synapsin I spots in these cultures. (**c**) Quantitation of the FM1-43 dots in wild-type and *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ (ki/ki) cultures after 8, 10 and 17 days. $^{**}p < 0.01$ (*t*-test). (**d**) Representative traces of spontaneous excitatory synaptic currents from mixed cultures of hippocampal neurons demonstrating the increased frequency of spontaneous synaptic currents recorded from *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ neurons. (**e**) Plot of FM4-64 puncta per cell versus the frequency of spontaneous quantal events. Increased numbers of FM puncta in *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ (wild type, 56.4 ± 4.7, $n = 5$; *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ 96.5 ± 7.0, $n = 6$; $^{**}p < 0.005$) are associated with increased levels of spontaneous neurotransmitter release (wild type 0.5 ± 0.1Hz, $n = 5$; *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ 3.2 ± 1.0Hz, $n = 6$; $^{*}p < 0.05$). (**f**) Exogeneous NT4 and BDNF specifically increased the density of FM1-43 punctae in cultured hippocampal neurons. Newborn wild-type hippocampal neurons were treated with NT4 and BDNF at 1–300 ng per ml for nine days, then stained for FM1-43 and synapsin I. Note that both NT4 and BDNF increased the density of FM1-43 dots in a dose-dependent fashion. However, the density of FMI-43 dots was significantly higher in NT4-treated cultures than those treated with BDNF at 100 ng per ml or greater. $^{*}p < 0.05$ between untreated control (0) and all other NT4- and BDNF- treated groups, or between NT4- and BDNF-treated groups at 100 and 300 ng per ml concentrations. (**g**) Comparison of densities of FM1-43 and synapsin I in cultures treated with 300 ng per ml of NT4 or BDNF for 9 days. $^{*}p < 0.001$ compared with untreated control cultures, $^{**}p < 0.01$ compared with BDNF-treated cultures (*t*-test).

than in wild-type cultures, indicating a higher number of functional synapses (**Fig. 3**). Although the density of FM punctae was 64% lower than that of synapsin I-immunoreactive sites in wild-type neurons, *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ neuronal cultures showed similar densities of FM dots and synapsin I spots (**Fig. 3b**). This finding suggests that knock-in NT4 specifically promoted the development of endocytosis-competent synapses in *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ cultures. Heterozygous *Bdnf* $^{+/nt4\text{-}ki}$ cultures had an intermediate increase in the FM density, consistent with a dosage effect of NT4 on synapse development (**Fig. 3b**). Knock-in NT4 also affected the time course of synapse formation in cultured hippocampal neurons. FM dots were detected two days earlier in *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ cultures than in wild-type cultures (eight versus ten days; **Fig. 3c**). The density of FM punctae in *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ neurons reached peak levels after 10 days in culture, in contrast to wild-type neurons, in which FM staining peaked after 17 days in culture (**Fig. 3c**). This suggests that the earlier appearance of functional synapses in *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ cultures reflects an acceleration of synapse formation.

To determine if an increase in FM punctae correlated directly with synaptic function, we compared frequencies of spontaneous transmitter release between *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ and wild-type control neurons (marked by green fluorescent protein, see Methods) in mixed cultures. Indeed, *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ cells had a significantly increased frequency of spontaneous secretion when compared with control neurons grown under identical conditions (**Fig. 3d**). To more accurately compare the spontaneous release frequency between genotypes, we determined the number of FM punctae (stained with FM4-64 dye showing red fluorescence) on each recorded cell and plotted the mean frequency of spontaneous release versus the mean number of punctae (**Fig. 3e**). The difference in spontaneous activity was significant between genotypes (wild-type 0.5 ± 0.1 Hz, $n = 5$; *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ 3.2 ± 1.0 Hz, $n = 6$; $^{*}p < 0.05$), suggesting that increased FM dye

Table 2. Number of cranial sensory neurons in wild type, *Bdnf*$^{nt4\text{-}ki/-}$; *trkB*$^{shc/shc}$ and *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$; *trkB*$^{shc/shc}$ mice.

	Wild type	Bdnf$^{nt4\text{-}ki/-}$; trkB$^{shc/sh}$		Bdnf$^{nt4\text{-}ki/nt4\text{-}ki}$; trkB$^{shc/shc}$	
Ganglion	Mean no. of neurons (contol)	Mean no. of neurons	% of control	Mean no. of neurons	% of control
Vestibular	6490 ± 213 (n = 8)	3234 ± 162$^{\#\#}$ (n = 4)	50 ± 2**	5096 ± 664$^{\#}$ (n = 2)	79 ± 6*
Nodose-petrosal	6859 ± 243 (n = 6)	1910 ± 168$^{\#\#}$ (n = 4)	28 ± 2**	3876 ± 44$^{\#\#}$ (n = 2)	57 ± 1**
Geniculate	1433 ± 38 (n = 6)	494 ± 33$^{\#\#}$ (n = 4)	34 ± 2**	676 ± 92$^{\#\#}$ (n = 2)	47 ± 6**

Counts are given as number ± s.e. $^{*}p < 0.05$, $^{**}p < 0.01$ (unpaired t-test, when compared with the wild-type control). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (unpaired t-test, when compared with the corresponding *Bdnf*$^{nt4\text{-}ki/-}$ or *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ groups in **Table 1**).

uptake reflected an increase in functional synaptic contacts. However, the amplitude of spontaneous events in the transgenic neurons was unaffected (wild type 7.2 ± 0.9 pA; *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$, 7.7 ± 1.3 pA; $p > 0.05$). Our data suggest that the substitution of NT4 for BDNF in these neurons increased the rate of functional synapse formation.

The enhanced effect of knock-in NT4 on functional-synapse formation could be due either to a unique property of NT4 or to a quantitative difference in NT4 and BDNF action on hippocampal neurons. To distinguish between these two possibilities, we examined whether both NT4 and BDNF, when added exogenously, can influence the appearance of FM punctae in wild-type hippocampal cultures. The density of FM punctae increased in a dose-dependent manner for either NT4 or BDNF (**Fig. 3f**), indicating that exogenous NT4 and BDNF each promoted synapse formation. Interestingly, although NT4 and BDNF were equally potent in promoting the development of synaptic contacts at low concentrations (≤10 ng per ml or 4×10^{-10} M), NT4 was far more effective at higher concentrations (**Fig. 3f**). Exogenous NT4 and BDNF did not influence the density of synapsin I-immunoreactive punctae, but specifically increased the FM punctae, consistent with what was observed in *Bdnf*$^{nt4\text{-}ki}$ neuronal cultures (**Fig. 3g**). Taken together, these data suggest that NT4, either expressed from the *Bdnf*$^{nt4\text{-}ki}$ allele or added exogenously, was more efficient than BDNF in promoting functional synapse formation.

The molecular mechanisms underlying the difference in activities *in vivo* between NT4 and BNDF is unclear. It is possible that NT4 and BDNF bind to TrkB and p75^{NTR} receptors slightly differently, leading to different receptor conformational changes and different strengths of downstream signaling. Indeed, a point mutation in the Shc binding site of the TrkB receptor (tyrosine 515) mainly affects the efficiency of NT4 signaling *in vivo* and *in vitro*, but only mildly affects BDNF[34]. To directly test whether the TrkB-Shc pathway is required for knock-in NT4 signaling, we introduced this TrkB point mutation (*trkB*^{shc}) into the *Bdnf*$^{nt4\text{-}ki}$ mice. Although occasionally hyperactive, *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$; *trkB*$^{shc/shc}$ mice survived into adulthood without dramatic behavioral abnormalities. In contrast, juvenile *Bdnf*$^{nt4\text{-}ki/-}$; *trkB*$^{shc/shc}$ mutants already show severe behavioral deficits similar to those of *Bdnf*$^{-/-}$ mice, including hyperactive spinning and head bobbing[22]. Nevertheless, *Bdnf*$^{nt4\text{-}ki/-}$; *trkB*$^{shc/shc}$ mice could survive into adulthood for four months or more, even though their '*Bdnf*$^{-/-}$-like' behavioral symptoms persisted throughout the entire lifespan. This suggests that the *Bdnf*$^{nt4\text{-}ki/-}$; *trkB*$^{shc/shc}$ phenotype was close to the *Bdnf*$^{-/-}$ phenotype, but not identical. Importantly, although *Bdnf*$^{+/-}$; *trkB*$^{shc/shc}$ mice tended to be obese in adulthood, they did not show behavioral deficits of *Bdnf*$^{nt4\text{-}ki/-}$; *trkB*$^{shc/shc}$ mice, suggesting that the TrkB-Shc signaling pathway is more crucial for NT4 signaling than for BDNF *in vivo*. Analysis of survival of sensory neurons in *Bdnf*$^{nt4\text{-}ki}$; *trkB*^{shc} compound-mutant mice confirmed that the TrkB-Shc pathway is more important for NT4 than for BDNF. Whereas *Bdnf*$^{+/+}$; *trkB*$^{shc/shc}$ mice do not show any obvious neuronal loss in vestibular ganglia at birth[34], neonatal *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$; *trkB*$^{shc/shc}$ mice did show a significant loss of vestibular neurons (**Table 2**). Consistent with their severe behavioral symptoms, sensory deficits in *Bdnf*$^{nt4\text{-}ki/-}$; *trkB*$^{shc/shc}$ were

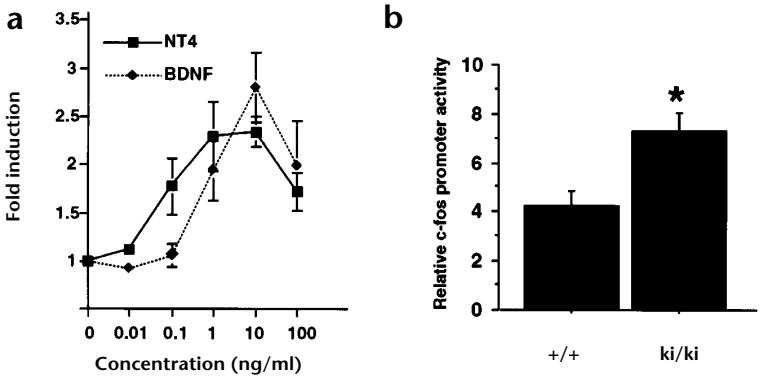

Fig. 4. NT4 is a more potent activator of the c-fos promoter than BDNF in mouse cortical cultures. (**a**) Dosage curve of NT4 and BDNF in activating a c-fos promoter. The induction of the c-fos promoter by NT4 and BDNF at each concentration was compared by paired t-tests (n = 4). Differences in the fold induction of the c-fos promoter were statistically significant ($p < 0.05$) when low concentrations of NT4 and BDNF (0.01, 0.1 and 1 ng per ml) were applied. (**b**) Activation of the c-fos promoter in wild type and *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ cortical cells. E16 wild-type (+/+, n = 6) and *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ (ki/ki, n = 12) neurons were transfected with both luciferase vectors described in (**a**) and harvested 24 h later for dual luciferase assays. The relative c-fos promoter activity is represented as a ratio of c-fos-fly luciferase activity over the control renilla luciferase activity. $^{*}p < 0.05$ (unpaired t-test).

much greater than in either the wild-type or *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$; *trkB*$^{shc/shc}$ mice (**Table 2**). Compared with NT4 knock-in mice carrying the wild-type TrkB receptor, the *trkB*$^{shc/shc}$ mutation significantly reduced the number of cranial sensory neurons (–33% to –70%; **Tables 1** and **2**). Thus, the TrkB-Shc signaling pathway is more important for NT4's survival activity *in vivo*.

Our results suggest that NT4 is more effective than BDNF in promoting neuronal survival and synapse formation. To reveal the intracellular signaling events that may underlie the different effects of NT4 and BDNF, we examined the activation of one of the target genes in the TrkB signaling cascade, the immediate-early gene c-fos. Both NT4 and BDNF induce c-fos expression in cortical neuronal cultures[35,36]. We therefore used a c-fos promoter-luciferase reporter to determine the dose–response curves for NT4 and BDNF on activation of transcription of this c-fos promoter. At low concentrations (0.01–1 ng per ml), NT4 was more potent than BDNF in activating a transfected c-fos promoter in cultured mouse cortical neurons (**Fig. 4a**). Moreover, this c-fos promoter was more active in cultured *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ cortical neurons than in wild-type cells (**Fig. 4b**). These results suggest that, NT4 and BDNF may differentially activate the trkB signaling cascade, at least at the level of inducing immediate-early genes.

Discussion

Although BDNF- and NT4-mutant mice showed non-overlapping neuronal deficits, our results demonstrated that when NT4 expression was under *Bdnf* gene regulatory control, NT4 rescued BDNF-deficient mice and supported many BDNF-dependent neurons *in vivo*. Thus, the contrasting phenotypes of BDNF- and NT4-deficient mice stem mainly from their divergent expression patterns.

Nevertheless, homozygous NT4 knock-in mice developed a distinct neurological phenotype, also suggesting certain unique activities for BDNF and NT4 *in vivo*. Indeed, our results showed that NT4 has a more potent activity than BDNF in promoting survival of sensory neurons and formation of hippocampal synapses. Therefore we examined the possibility that a divergence in TrkB receptor signaling might be responsible for different activities of NT4 and BNDF. We demonstrated that in *Bdnf*$^{nt4\text{-}ki}$ neurons and wild-type cells, a transfected c-fos promoter could be differentially activated, providing evidence in supporting of a possible difference between NT4 and BDNF in strength of activation of downstream TrkB signaling.

Although both BDNF and NT4 can activate many common signaling pathways for neuronal survival[12,36], the TrkB receptor seems capable of distinguishing between the binding of NT4 or BDNF *in vivo*. For example, splice variants of the TrkB receptor can have different specificities for BDNF and NT4 binding[40]. In addition, a point mutation in the TrkB receptor (Y515F, *trkB*^{shc}) can lead to distinctive intracellular responses to NT4 and BDNF activation[34]. The *trkB*^{shc} mutation differentially shifts the dose–response curves for NT4's and BDNF's support of sensory neurons: NT4 signaling is more affected than BDNF by this mutation (see figure 6 of ref. 34). By introducing this intracellular *trkB*^{shc} point mutation into the NT4 knock-in mice, we confirmed that the TrkB-Shc pathway is more crucial for knock-in NT4 than for endogenous BDNF in promoting neuronal survival. Moreover, homozygous *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$;*trkB*$^{shc/shc}$ mice show much lower neuronal loss than *Bdnf*$^{nt4\text{-}ki/-}$; *trkB*$^{shc/shc}$ mutants, also indicating an NT4-dosage effect in the presence of the *trkB*^{shc} mutation. As the extracellular domain of the TrkB receptor for NT4 and BDNF binding is unaltered, the different effect of the *trkB*^{shc} mutation for NT4 and BDNF must be mediated by the differences in intracellular signaling. Taken together, our evidence supports the notion that a divergence of TrkB receptor signaling could partly underlie the distinct activities of NT4 and BDNF *in vivo*.

Previous evidence indicates that p75^{NTR} can either increase or decrease the efficiency of TrkB activation by NT4 and BDNF *in vitro* in different culture systems[24,37]. In addition, p75^{NTR} is more important for the retrograde transport of NT4 than for BDNF *in vivo*[38]. These results suggest that p75^{NTR} may be another determinant underlying the different activities of NT4 and BDNF. NT3 and BDNF signaling is also potentiated by p75^{NTR} during proprioceptive sensory development (G.F. *et al.*, *Soc. Neurosci. Abstr.*, **25**, 707.5, 1999; ref. 39). Whether p75^{NTR} is required for efficient TrkB activation by NT4 *in vivo* can be addressed by introducing p75^{NTR} mutations into *Bdnf*$^{nt4\text{-}ki}$ mice.

Our data demonstrate that NT4, either replacing the endogenous BDNF or added exogenously, promotes the formation of functional synapses between newborn hippocampal neurons as shown by increases in endocytotic dye uptake and spontaneous transmitter-release frequency. Exogenous BDNF (or NT3) enhances spontaneous transmitter release in fetal hippocampal neurons at the midgestational stage[41]. Our results extend previous findings and suggest that the increased spontaneous release may be related to the precocious appearance of FM punctae at the existing synapses. The accelerated formation of functional synapse sites indicates that NT4/TrkB signaling is sufficient in inducing functional changes in synapses without influencing the total number of synaptic contacts. A major benefit of the culture system used here is that the coordinated process of synapse formation and maturation can be examined over time. It will be of interest to examine whether knock-in NT4 modulates the time course of functional synapse formation and influences synaptic functions *in vivo*, considering that endogenous BDNF plays a role in the synaptic plasticity of hippocampal neurons[29–31].

Here we demonstrated that knocking NT4 into the BDNF locus had a gene-dosage effect on survival of sensory neurons and formation of functional synapses in hippocampal neurons. This gene-dosage effect was observed in the presence of either wild-type TrkB or mutated TrkB^{shc} receptors. This result contrasts with the finding that heterozygous mutants for the endogenous NT4 show no significant sensory deficits[25], indicating the lack of a NT4 gene-dosage effect. At least two possibilities could explain this lack of a gene-dosage effect for the endogenous NT4 gene. First, regulation of the endogenous NT4 gene may be different from other neurotrophin genes, and heterozygous NT4 mutants may maintain endogenous NT4 mRNA or protein levels at wild-type levels via some form of compensation feedback. Second, the endogenous NT4 level is low compared to levels of endogenous BDNF or knock-in NT4, and may be below the dose response curve. Therefore, even though there may be a 50% reduction of endogenous NT4 in NT4 heterozygous mutants, it is not enough to affect survival of sensory neurons.

The replacement of BDNF by NT4 seems to affect the function of many neural systems, including the cutaneous sensory system and the neural components involved in reproduction and control of body weight. The specific neuronal changes that underly these phenotypes are still unknown. The body weight loss in NT4 knock-in mice is in contrast to the phenotype of BDNF heterozygous mice, which are chronically hyperphagic and obese[42]. This obese phenotype is attributed to the dysfunction of central 5-HT neurons, which have altered expression of 5-HT receptors, reduced 5-HT levels and blunted c-fos induction as a consequence of reduced BDNF levels[42]. Perhaps the reduced body

weight in NT4 knock-in mice was due to the over-activation of TrkB signaling *in vivo* by NT4, as we showed that NT4 was more active than BDNF for neuronal survival, synapse formation and activation of the c-fos promoter. Nevertheless, we cannot rule out the possibility that NT4 has a unique biological activity that contributes to this phenotype. Previous studies from NT4 and BDNF mutant mice suggest that NT4 and BDNF are required for different classes of skin sensory fibers[43,44]. In addition, evidence exists that NT4 and BDNF can play different roles in a variety of events including modulating dendritic arborization, dopamine uptake, reversing spatial memory impairment and neuronal migration[45–48]. The generation of NT4 knock-in mice certainly provides a useful model with which to address in detail the distinct functions of BDNF and NT4 *in vivo*.

Methods

***Bdnf*$^{nt4\text{-}ki}$ and *trkB*^{shc} mice.** *Bdnf*$^{nt4\text{-}ki}$ mice were generated through gene targeting in ES cells and blastocyst injections of targeted ES cells. The generation of *trkB*^{shc} mice was as reported[34]. Southern blot analysis and PCR reactions were used for genotyping mice.

Northern blots. Total RNAs were extracted from brain tissues using a RNAzol kit (Tel-Test, Friendwood, Texas), subjected to electrophoresis and transferred to a nylon membrane. The blot was hybridized using the standard Quickhyb protocol (Stratagene, La Jolla, California).

NT4 and BDNF Elisa. Fresh tissues dissected from adult mice were homogenized by polytron and extracted in twenty-volumes of a high salt lysis buffer. Extracted supernatants were subject to Elisa analysis using the standard protocols in the commercial NT4 and BDNF Elisa kits (Promega, Madison, Wisconsin).

Histology and neuron counts. Tissues dissected from newborn or adult mice were fixed in 4% paraformaldehyde/PBS for 24 h, embedded in OCT for frozen sections, or processed with a VIP tissue processor (Miles) for paraffin sectioning. For neuronal counts in cranial sensory ganglia, heads from newborn mice were sectioned at 5 μm thickness, and serial sections were stained with cresyl violet. Neurons with a clear nucleus and nucleoli were counted in every eighth section. Total numbers of neurons in sensory ganglia were corrected as described[20].

Cell cultures, plasmid transfection and luciferase assays. Hippocampi and cortices were dissected from newborn and embryonic mice, treated with trypsin, dissociated and plated on coated-glass coverslips and were cultured as previously described[49,50]. For mixed cultures, approximately equal numbers of *Bdnf*$^{nt4\text{-}ki/nt4\text{-}ki}$ and wild-type (derived from the GFP transgenic mice, kindly provided by M. Okabe, Japan) hippocampal neurons were seeded. Transfection of plasmids [c-fos-promoter $^{(-711\ to\ +109)}$-fly luciferase construct (a gift from R. Misra) and pRL-TK renilla-luciferase vector (Promega)] was performed as described previously[50]. Wild-type E16–17 mouse cortical cultures were transfected with a c-fos-promoter/fly-luciferase reporter construct and pRL-TK renilla luciferase control vectors after one day *in vitro*. Transfected cultures were treated with different dose of NT4 or BDNF for 12 h. Cells were extracted and subjected to dual luciferase assays. After normalization for the transfection efficiencies, the fold induction of the relative luciferase activities by NT4 or BDNF over untreated controls was plotted. Dual-luciferase assays was performed with a commercial reagent (Promega).

Immunocytochemistry. Tissue sections or cell cultures were fixed with 4% paraformaldehyde and permeabilized with Triton X-100. Specific primary antibodies were applied and visualized with fluorescein conjugated secondary antibodies.

FM staining and counting. Cultures were stained with 10 μM FM1-43 or FM4-64 dye (Molecular Probes, Eugene, Oregon) in 90 mM [K^+] for 1 min, then washed for >5 min in Tyrode solution: 128 mM NaCl, 5 mM KCl, 30 mM glucose, 25 mM HEPES, 1 mM $MgCl_2$, 2 mM $CaCl_2$ plus 1 μM tetrodotoxin (TTX; Oretek, Freemont, California). Imaging was captured with an (Olympus, Melville, New York) Fluoview scanning laser confocal microscope. The number of FM punctae per 20 μm was determined by manually counting the punctae on 400 dendritic segments taken from 10 different regions from at least 2 different cultures.

Electrophysiology. Techniques for whole-cell recording from cultured hippocampal neurons have been reported previously[49]. Spontaneous quantal release frequencies were assayed during a ten minute period of continuous recording at a holding potential of –60 mV. Recordings were made with a 200B integrating patch clamp amplifier (Axon Instruments, Foster City, California) with a 1 kHz (8 pole Bessel) low-pass filter. Data were digitized at 10 kHz using a Digidata 1200B A/D converter (Axon Instruments) and analyzed with in-house software.

Acknowledgements

We would like to thank Brian Bates, Jeffrey Cottrell, Scott Waddell and Michael Greenberg for reading the manuscript. We appreciate the technical support from Jesse Dausemann, Ruth Curry and Jeanne Reis. This work was supported by NIH grants (R.J. and G.L.), Deutsche Forschungsgemeinschaft and the European Union (R.K.), a Medical Foundation Postdoctoral Fellowship (G.F.), and RIKEN-MIT fellowships (C.E. and J.J.R.).

RECEIVED 14 JANUARY; ACCEPTED 16 FEBRUARY 2000

1. Lewin, G. R. & Barde, Y. A. Physiology of the neurotrophins. *Annu. Rev. Neurosci.* **19**, 289–317 (1996).
2. McAllister, A. K., Katz, L. C. & Lo, D. C. Neurotrophins and synaptic plasticity. *Annu. Rev. Neurosci.* **22**, 295–318 (1999).
3. Levi-Montalcini, R. The nerve growth factor 35 years later. *Science* **237**, 1154–1162 (1987).
4. Leibrock, J. *et al.* Molecular cloning and expression of brain-derived neurotrophic factor. *Nature* **341**, 149–152 (1989).
5. Hohn, A., Leibrock, J., Bailey, K. & Barde, Y. A. Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. *Nature* **344**, 339–341 (1990).
6. Maisonpierre, P. C. *et al.* Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. *Science* **247**, 1446–1451 (1990).
7. Rosenthal, A. *et al.* Primary structure and biological activity of a novel human neurotrophic factor. *Neuron* **4**, 767–773 (1990).
8. Ernfors, P., Ibanez, C. F., Ebendal, T., Olson, L. & Persson, H. Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in the brain. *Proc. Natl. Acad. Sci. USA* **87**, 5454–5458 (1990).
9. Jones, K. R. & Reichardt, L. F. Molecular cloning of a human gene that is a member of the nerve growth factor family. *Proc. Natl. Acad. Sci. USA* **87**, 8060–8064 (1990).
10. Berkemeier, L. R. *et al.* Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. *Neuron* **7**, 857–866 (1991).
11. Ip, N. Y. *et al.* Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. *Proc. Natl. Acad. Sci. USA* **89**, 3060–3064 (1992).
12. Barbacid, M. The Trk family of neurotrophin receptors. *J. Neurobiol.* **25**, 1386–1403 (1994).
13. Bothwell, M. Functional interactions of neurotrophins and neurotrophin receptors. *Annu. Rev. Neurosci.* **18**, 223–253 (1995).
14. Snider, W. D. Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. *Cell* **77**, 627–638 (1994).
15. Klein, R. *et al.* The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. *Cell* **66**, 395–403 (1991).
16. Klein, R., Lamballe, F., Bryant, S. & Barbacid, M. The trkB tyrosine protein kinase is a receptor for neurotrophin-4. *Neuron* **8**, 947–956 (1992).
17. Chao, M. V. The p75 neurotrophin receptor. *J. Neurobiol.* **25**, 1373–1385 (1994).
18. Davies, A. M., Lee, K. F. & Jaenisch, R. p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. *Neuron* **11**, 565–574 (1993).
19. Ibanez, C. F. Neurotrophin-4: the odd one out in the neurotrophin family. *Neurochem. Res.* **21**, 787–793 (1996).
20. Liu, X., Ernfors, P., Wu, H. & Jaenisch, R. Sensory but not motor neuron deficits in mice lacking NT4 and BDNF. *Nature* **375**, 238–241 (1995).
21. Conover, J. C. *et al.* Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. *Nature* **375**, 235–238 (1995).
22. Ernfors, P., Lee, K. F. & Jaenisch, R. Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature* **368**, 147–150 (1994).

23. Jones, K. R., Farinas, I., Backus, C. & Reichardt, L. F. Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. *Cell* **76**, 989–999 (1994).
24. Bibel, M., Hoppe, E. & Barde, Y. A. Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. *EMBO. J.* **18**, 616–622 (1999).
25. Erickson, J. *et al.* Mice lacking brain-derived neurotrophic factor exhibit visceral sensory neuron losses distinct from mice lacking NT4 and display a severe developmental deficit in control of breathing. *J. Neurosci.* **16**, p5361–5371 (1996)
26. ElShamy, W. M. & Ernfors, P. Brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4 complement and cooperate with each other sequentially during visceral neuron development. *J. Neurosci.* **17**, 8667–8675 (1997).
27. Bianchi, L. M. *et al.* Degeneration of vestibular neurons in late embryogenesis of both heterozygous and homozygous BDNF null mutant mice. *Development* **122**, 1965–1973 (1996).
28. Schwartz, P. M., Borghesani, P. R., Levy, R. L., Pomeroy, S. L. & Segal, R. A. Abnormal cerebellar development and foliation in BDNF$^{-/-}$ mice reveals a role for neurotrophins in CNS patterning. *Neuron* **19**, 269–281 (1997).
29. Korte, M. *et al.* Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. *Proc. Natl. Acad. Sci. USA* **92**, 8856–8860 (1995).
30. Patterson, S. L. *et al.* Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron* **16**, 1137–1145 (1996).
31. Pozzo-Miller, L. D. *et al.* Impairments in high-frequency transmission, synaptic vesicle docking, and synaptic protein distribution in the hippocampus of BDNF knockout mice. *J. Neurosci.* **19**, 4972–4983 (1999).
32. Fletcher, T. L., De Camilli, P. & Banker, G. Synaptogenesis in hippocampal cultures: evidence indicating that axons and dendrites become competent to form synapses at different stages of neuronal development. *J. Neurosci.* **14**, 6695–6706 (1994).
33. Ryan, T. A. *et al.* The kinetics of synaptic vesicle recycling measured at single presynaptic boutons. *Neuron* **11**, 713–724 (1993).
34. Minichiello, L. *et al.* Point mutation in trkB causes loss of NT4-dependent neurons without major effects on diverse BDNF responses. *Neuron* **21**, 335–345 (1998).
35. Ghosh, A., Carnahan, J. & Greenberg, M. E. Requirement for BDNF in activity-dependent survival of cortical neurons. *Science* **263**, 1618–1623 (1994).
36. Segal, R. & Greenberg, M.E.. Intracellular signaling pathways activated by neurotrophic factors. *Ann. Rev. Neurosci.* **19**, 463–489 (1996).
37. Ryden, M. *et al.* Functional analysis of mutant neurotrophins deficient in low-affinity binding reveals a role for p75LNGFR in NT-4 signalling. *EMBO. J.* **14**, 1979–1990 (1995).
38. Curtis, R. *et al.* Differential role of the low affinity neurotrophin receptor (p75) in retrograde axonal transport of the neurotrophins. *Neuron* **14**, 1201–1211 (1995).
39. Fan, G., Jaenisch, R. & Kucera, J. A role for p75 receptor in neurotrophin-3 functioning during the development of limb proprioception. *Neuroscience* **90**, 259–268 (1999).
40. Strohmaier, C., Carter, B., Urfer, R., Barde, Y. & Dechant, G. A splice variant of the neurotrophin receptor trkB with increased specificity for brain-derived neurotrophic factor. *EMBO J.* **15**, 3332–3337 (1996).
41. Vicario-Abejon, C., Collin, C., McKay, R. D. & Segal, M. Neurotrophins induce formation of functional excitatory and inhibitory synapses between cultured hippocampal neurons. *J. Neurosci.* **18**, 7256–7271 (1998).
42. Lyons, W. E. *et al.* Brain-derived neurotrophic factor-deficient mice develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities. *Proc. Natl. Acad. Sci. USA* **96**, 15239–15244 (1999)
43. Stucky, C., De, C. T., Lindsay, R., Yancopoulos, G. & Koltzenburg, M. Neurotrophin 4 is required for the survival of a subclass of hair follicle receptors. *J. Neurosci.* **18**, 7040–7046 (1998).
44. Carroll, P., Lewin, G. R., Koltzenburg, M., Toyka, K. V. & Thoenen, H. A role for BDNF in mechanosensation. *Nat. Neurosci.* **1**, 42–46 (1998).
45. McAllister, A. K., Lo, D. C. & Katz, L. C. Neurotrophins regulate dendritic growth in developing visual cortex. *Neuron* **15**, 791–803 (1995).
46. Hyman, C. *et al.* Overlapping and distinct actions of the neurotrophins BDNF, NT-3, and NT-4/5 on cultured dopaminergic and GABAergic neurons of the ventral mesencephalon. *J. Neurosci.* **14**, 335–347 (1994).
47. Fischer, W., Sirevaag, A., Wiegand, S. J., Lindsay, R. M. & Bjorklund, A. Reversal of spatial memory impairments in aged rats by nerve growth factor and neurotrophins 3 and 4/5 but not by brain-derived neurotrophic factor. *Proc. Natl. Acad. Sci. USA* **91**, 8607–8611 (1994).
48. Brunstrom, J. E., Gray-Swain, M. R., Osborne, P. A. & Pearlman, A. L. Neuronal heterotopias in the developing cerebral cortex produced by neurotrophin-4. *Neuron* **18**, 505–517 (1997).
49. Liu, G. & Tsien, R. W. Properties of synaptic transmission at single hippocampal synaptic boutons. *Nature* **375**, 404–408 (1995).
50. Bonni, A. *et al.* Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. *Science* **278**, 477–483 (1997).