TrkB and TrkC neurotrophin receptors cooperate in promoting survival of hippocampal and cerebellar granule neurons

Liliana Minichiello and Rüdiger Klein¹

European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69117 Heidelberg, Germany

The Trk family of protein tyrosine kinases (TrkA/B/C) are receptors for neurotrophins, a family of closely related proteins that are important physiological regulators of the survival of specific neurons within the peripheral nervous system (PNS) of vertebrates. In contrast to the PNS, brains of mutant mice deficient in a single neurotrophin or Trk receptor species do not show signs of major cell loss. However, in double mutant mice, we now show that reducing the expression of both TrkB and TrkC causes massive cell death of postnatal hippocampal and cerebellar granule neurons. Kinetic analysis of neuronal death in the hippocampus showed that dentate gyrus granule neurons become dependent on TrkB and TrkC after the first postnatal week, shortly after the period of naturally occurring cell death, indicating a role of these receptors in supporting postmitotic neurons. Correlating with the loss of granule cells, the number of mossy fibers projecting to CA3 pyramidal neurons was markedly reduced in mice carrying mutant *trkB/trkC* alleles, demonstrating impairment of excitatory pathways in the hippocampus. In the cerebellum, TrkB and TrkC receptors were specifically required for premigratory granule neurons located in the external granule layer. In contrast, cerebellar Purkinje cells were found to be poorly differentiated, but showed no signs of increased cell death. These results provide *in vivo* evidence that neurotrophins are essential physiological survival factors for specific central neurons. Moreover, they suggest that central, in contrast to peripheral, neurons are capable of using more than one neurotrophin/Trk receptor signaling pathway to stay alive.

[**Key Words:** Neurotrophins; Trk; receptor tyrosine kinase; hippocampus; dentate gyrus; cerebellum; apoptosis]

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Limited amounts of neurotrophic factors are thought to be the key determinants to regulate the number of surviving neurons during development. The best-studied family of neuronal survival factors are the neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), NT4/5, and NT6, the latter apparently being specific for fish (Götz et al. 1994; Lewin and Barde 1996). The effects of neurotrophins are primarily mediated by protein-tyrosine kinase receptors of the Trk family, with TrkA being a receptor for NGF, TrkB being a receptor for both BDNF and NT4/5, and TrkC being the preferred receptor for NT3, which can also interact with lower efficiency with TrkA and TrkB (Davies et al. 1995). Recent studies in neurotrophin and Trk receptor knockout mice have revealed their critical functions during neural development. One important conclusion from these genetic experiments was that many peripheral neurons depend for

their survival on a supply of a single type of neurotrophin from the tissues they innervate (Davies 1994a; Klein 1994; Snider 1994; Barbacid 1995). In contrast, although previous studies have demonstrated survival-promoting effects of exogenous neurotrophins on central nervous system (CNS) neurons (Eide et al. 1993; Henderson et al. 1993; Arenas and Persson 1994), essentially all neurotrophin-responsive CNS neurons appear to survive in absence of a single neurotrophin or its cognate receptor (Farinas and Reichardt 1996; Henderson 1996). It is therefore currently hypothesized that, unlike peripheral neurons, central neurons may have access to other neurotrophic factors that can compensate for these deficiencies *in vivo*. Considering that neurotrophins have been implicated as regulators of neuronal differentiation and as modulators of synaptic efficacy (Cabelli et al. 1995; Cohen-Cory and Fraser 1995; Kang and Schuman 1995; Thoenen 1995), it is even conceivable that neurotrophins do not have a physiological role in the survival of CNS neurons and serve only as survival factors for peripheral neurons.

¹Corresponding author.

To investigate whether two Trk receptors may have overlapping functions in promoting central neuron survival, we crossed mice carrying loss-of-function alleles of different *trk* genes (Klein et al. 1993, 1994; Smeyne et al. 1994) to generate mice doubly deficient in pairs of two different Trk receptors. Here we show that during the second postnatal week, TrkB and TrkC receptors are required to prevent apoptosis of at least two CNS neuron populations, hippocampal dentate gyrus and cerebellar granule neurons. In contrast to their action on peripheral neurons, TrkB and TrkC function in a compensatory, redundant fashion indicating that CNS neurons can use more than one neurotrophin/Trk receptor signaling pathway for survival.

Results

*Increased number of pyknotic cells in the dentate gyrus of mice carrying mutant *trkB* and *trkC* alleles*

None of the double homozygous (*trkA*^{-/-};*trkB*^{-/-}, *trkA*^{-/-};*trkC*^{-/-}, and *trkB*^{-/-};*trkC*^{-/-} mice resulting from crosses of corresponding heterozygotes survived beyond postnatal day 1 (P1). We therefore concentrated our analysis on mice that were homozygous for one mutant *trk* allele, and heterozygous mutant for a second Trk receptor, for example *trkB*^{-/-};*trkC*^{+/-}, a considerable fraction of which survived until the end of the second postnatal week. Those genotypes are likely to reveal dosage-dependent genetic interaction between Trk receptor, because their expression is reduced by approximately half in heterozygotes (Klein et al. 1993, 1994). As a morphological hallmark of cells undergoing programmed cell death, we counted pyknotic nuclei, which were recognized either as darkly stained spherical structures with pale or absent cytoplasm or by the presence of darkly stained granules of nuclear chromatin. Analysis of forebrains of P12 double mutant mice revealed a dramatic accumulation of pyknotic nuclei specifically in the hippocampal dentate gyrus of mice carrying mutant *trkB* and *trkC* alleles (Fig. 1). Whereas among the single mutants, only *trkB*^{-/-} mice showed a small (less than twofold) yet significant increase of pyknotic nuclei in the granule cell layer of the dentate gyrus, there were a 14- and 10-fold increase in *trkB*^{-/-};*trkC*^{+/-} and *trkB*^{+/-};*trkC*^{-/-} mice, respectively, compared with wild-type mice (Fig. 2A). In contrast, double heterozygous *trkB*^{+/-};*trkC*^{+/-} mice behaved like wild-type mice (data not shown). Moreover, all combinations of double mutant mice involving the mutant *trkA* allele (*trkA*^{-/-};*trkB*^{+/-}, *trkA*^{+/-};*trkB*^{-/-}, *trkA*^{-/-};*trkC*^{+/-}, *trkA*^{+/-};*trkC*^{-/-}) showed a small, approximately twofold, increase in pyknotic nuclei compared with wild-type mice, a value that was in most cases not significantly higher than in their corresponding single mutant mice. The observed pyknotic cells are likely to be granule neurons, because glial cells are rare in the granule cell layer of the dentate gyrus (Kosaka and Hama 1986; Rickmann et al. 1987). These findings indicate that TrkB and TrkC re-

Figure 1. Morphology of pyknotic cells in the hippocampal dentate gyrus of *trk* double mutant mice. Representative Nissl-stained 40- μ m coronal sections of the granule cell layer of the dentate gyrus of a P12 wild-type (A) and a *trkB*^{-/-};*trkC*^{+/-} mutant mouse (B). Note the typical appearance of pyknotic cells (arrowheads) in panel B with darkly stained condensed chromatin. Magnification, 1000 $\times$.

ceptors cooperate in promoting the survival of dentate gyrus neurons in the postnatal brain.

Dentate gyrus neurons require TrkB and TrkC after the period of naturally occurring cell death

The majority of hippocampal granule cells are generated after birth in rodents and the peak of naturally occurring neuronal cell death occurs toward the end of the first postnatal week (Gould et al. 1991). We therefore characterized the kinetics of neuronal cell death in mice carrying mutant *trkB* and *trkC* alleles between birth and P12. At birth, the numbers of pyknotic cells were low in wild-type and all mutant mice analyzed (data not shown). At P6, the numbers of pyknotic cells within the dentate gyrus increased to similar levels in wild-type and *trkB*/*trkC* mutant mice [1032 ± 123 ($n=3$) and 1027 ± 108 ($n=5$), respectively]. At P9, *trkB*^{-/-};*trkC*^{+/-} and *trkB*^{+/-};*trkC*^{-/-} mutant mice showed a significant, approximately fourfold increase in pyknotic nuclei compared with wild-type mice, whereas no such increase was observed in the single mutants (Fig. 2B). These results demonstrate that survival of dentate gyrus granule cells requires expression of TrkB and TrkC receptors shortly after the period of naturally occurring neuronal cell death.

Hippocampal dentate gyrus neurons undergo apoptosis

Because pyknotic nuclei are phagocytized within minutes, we employed a more sensitive method for the de-

Figure 2. (A) Comparison of the number of pyknotic cells in the dentate gyrus of P12 wild-type (+/+), single *trkA* -/- (A-/-), *trkB* -/- (B-/-), *trkC* -/- (C-/-), and double mutant mice of the indicated genotype (e.g., B-/-; C+/- for *trkB* -/-; *trkC* +/-. The data were obtained from three to four mice of all genotypes. Values represent means and standard errors per dentate gyrus on hemisections of P12 mice. Statistical analysis was carried out using Student's *t*-test and is indicated where significant. (*) $P < 0.01$; (**) $P < 0.001$. (B) Increase in number of pyknotic cells in mutant compared with wild-type dentate gyri in brains of P6, P9, and P12 mice. Data from *trkB* -/-; *trkC* +/+ and *trkB* +/--; *trkC* -/- mice were combined. Each bar represents the means ($\pm$ S.E.) from four to seven animals.

tection of apoptotic cells, known as TUNEL staining, which is based on nick end-labeling of DNA fragments in situ (Gavrielli et al. 1992). As shown in Figure 3, TUNEL staining of *trkB* -/-; *trkC* +/+ and *trkB* +/--; *trkC* -/- brains showed a massive amount of apoptotic cells in the hippocampal dentate gyrus. Quantitative analysis revealed a very good correlation with the scores obtained from cresyl violet-stained sections (data not shown). In addition, scattered TUNEL-positive cells were observed in superficial layers of the cerebral cortex, striatum, thalamus, and hypothalamus. Interestingly, few if any TUNEL-positive cells were observed in the pyramidal layer of the hippocampus. In wild-type, *trkB* -/- and *trkC* -/- brains, TUNEL-positive cells

were very rare, correlating well with the number of pyknotic cells in Nissl-stained sections. Combinations involving *trkA* mutant alleles together with either *trkB* or *trkC* did not result in significantly more apoptotic cells than in corresponding single mutants, neither in the dentate gyrus (Fig. 3) nor elsewhere in the brain (data not shown). These results demonstrate that, at least during early postnatal life, dentate gyrus neurons are most vulnerable to the lack of neurotrophin signaling compared with other forebrain neuron populations. Analysis by transmission electron microscopy revealed typical apoptotic granule cells with large areas of condensed nuclear chromatin in the brains of *trkB* -/-; *trkC* +/+ mice (data not shown). These results indicate that hip-

Figure 3. Increase in the number of TUNEL-positive dentate gyrus neurons in mice carrying mutant *trkB* and *trkC* alleles. Coronal sections from P12 wild-type (A), *trkB* -/-; *trkC* +/+ (B,C), *trkB* +/--; *trkC* -/- (D), *trkA* +/--; *trkB* -/- (E), *trkA* +/--; *trkC* -/- mice (F) stained by the TUNEL technique. Note the massive amount of TUNEL-positive cells specifically in the hippocampal dentate gyrus in B-D (the shape of the dentate gyrus is outlined by arrowheads in A and B). Pyramidal neurons of the hippocampus (CA1 through CA3 regions) lack significant numbers of TUNEL-positive cells. Scattered TUNEL-positive cells are seen in superficial layers of the somatosensory cortex (sc), striatum (s), thalamus (t), and hypothalamus (ht). Magnification: (A,B) 25 $\times$; (C-F) 1000 $\times$.

pocampal granule cells of *trkB/trkC* double mutant mice undergo programmed cell death.

*Reduced numbers of excitatory mossy fibers in the hippocampus of mice carrying mutant *trkB* and *trkC* alleles*

Dentate gyrus granule cells form excitatory connections, so-called mossy fibers, with CA3 pyramidal neurons, a pathway that is implicated in certain forms of long-term potentiation (Zalutsky and Nicoll 1990). As an indirect measure of function of the remaining viable granule cells in mice carrying mutant *trkB* and *trkC* alleles, we estimated the amount of mossy fibers projecting from the dentate gyrus to CA3 pyramidal neurons. We immunostained coronal sections for the Ca^{2+} binding protein calbindin (Celio 1990), which is a molecular marker for dentate gyrus granule cells and mossy fibers. The pattern of calbindin staining in single *trkB*^{−/−} and *trkC*^{−/−} mutant mossy fibers was indistinguishable from that in wild-type mice (Fig. 4 and data not shown). In contrast, both double *trkB*^{−/−}; *trkC*^{+/−} (Fig. 4C) and *trkB*^{+/−}; *trkC*^{−/−} brains (not shown) reproducibly showed a

marked reduction in calbindin positive mossy fibers. Using an image analysis program for quantitative analysis (Olivo et al. 1993), the cross-sectional area of calbindin positive mossy fibers was significantly reduced in *trkB*^{−/−}; *trkC*^{+/−} (58%) and *trkB*^{+/−}; *trkC*^{−/−} (48%) brains, respectively, compared with wild-type, and ~40% compared with the single mutants (Table 1). Because the brains of *trkB*^{−/−}; *trkC*^{+/−} and *trkB*^{+/−}; *trkC*^{−/−} were not significantly smaller than those of the corresponding single mutants (data not shown), the reduction in mossy fibers is likely to be a consequence of the dramatic increase in cell death in the dentate gyrus. In contrast, the 20% reduction in cross-sectional area of mossy fibers in the single mutants is in part a result of the 20–25% smaller brain size of mutant versus wild-type brains. Mice carrying combinations of mutant *trkA* alleles with either mutant *trkB* or *trkC* alleles did not show a significant reduction of mossy fibers (data not shown). These results indicate that TrkB and TrkC receptors cooperate in maintaining the efferent projections of dentate granule cells to CA3 pyramidal neurons.

Figure 4. Reduced numbers of calbindin-positive mossy fibers projecting from dentate gyrus neurons in *trkB/trkC* double mutant mice. Coronal sections from wild-type (A), *trkB*^{−/−} (B), and *trkB*^{−/−}; *trkC*^{+/−} (C), immunostained with a calbindin-specific antibody. Note the reduction of cross-sectional area of mossy fibers (indicated by arrowheads) in *trkB*^{−/−}; *trkC*^{+/−} double mutants compared with wild-type and single mutant mice.

Cerebellar granule cells require TrkB and TrkC receptors for survival

Analysis of cresyl violet-stained sagittal sections of P12 cerebella derived from *trkB/trkC* double mutant mice revealed an enlargement of the external granule layer (EGL), a zone where granule cell precursors divide and undergo the initial phase of neuronal differentiation (see Fig. 7, below). Both *trkB*^{−/−}; *trkC*^{+/−} and *trkB*^{+/−}; *trkC*^{−/−} mice had 40 and 50% more EGL neurons, respectively, compared with wild-type mice (data not shown; $n=3$ for each genotype; $P<0.01$). In contrast, *trkB*^{−/−} and *trkC*^{−/−} single mutants showed a small, yet not significant increase in the numbers of EGL neurons compared with wild-type cerebella (data not shown). The numbers of pyknotic cells in the EGL of *trkB*^{−/−} and *trkC*^{−/−} single mutants were also slightly elevated compared with wild-type mice (Fig. 5). Surprisingly, the numbers of pyknotic cells in *trkB*^{−/−}; *trkC*^{+/−} and *trkB*^{+/−}; *trkC*^{−/−} double mutants were increased 7.5- and 6-fold, respectively, compared with wild-type mice and 5- and 5.5-fold, respectively, compared with *trkB*^{−/−} and *trkC*^{−/−} mice (Fig. 5). Likewise, TUNEL staining revealed a marked increase in apoptotic cells in sections derived from mice carrying mutant *trkB* and *trkC* alleles compared with wild-type or single mutants (Fig. 6 and data not shown). Interestingly, the internal granule layer (IGL), which contains fully differentiated granule cells after their migration through the molecular layer, did not contain significant numbers of TUNEL-positive or pyknotic cells in mice with mutant *trkB* and *trkC* alleles (data not shown). These results indicate that TrkB and TrkC receptors cooperate in promoting survival of premigratory cerebellar granule cells.

Table 1. Reduction of calbindin-positive mossy fibers in *trkB/trkC* double mutant mice

Genotype	No. of animals	Surface of calbindin ⁺ mossy fibers $\pm$ S.E.M. ($\times 1000 \mu\text{m}^2$)	Reduction compared with wild-type (%)	Reduction compared with single mutants (%)
Wild-type	3	126 $\pm$ 10		
<i>trkB</i> ^{-/-} or <i>trkC</i> ^{-/-}	4	102 $\pm$ 7	20 (n.s.)	
<i>trkB</i> ^{-/-} ; <i>trkC</i> ^{+/-}	4	53 $\pm$ 4	58 ^a	43 ^a
<i>trkB</i> ^{+/-} ; <i>trkC</i> ^{-/-}	3	66 $\pm$ 3	48 ^b	40 ^b

The cross-sectional area of calbindin-positive mossy fibers was measured using an image analysis program (Olivo et al. 1993), and the average value was taken from two hemisections per animal. Values represent mean $\pm$ standard errors from the indicated number of animals. Values for single mutant mice were combined. No statistical difference was observed between single mutants. Statistical analysis was carried out using Student's *t*-test. n.s.: not significant.

^a*P* < 0.001.

^b*P* < 0.01.

Redundant function of *TrkB* and *TrkC* receptors in Purkinje cell differentiation, but not survival

The second major cell type of the cerebellum, the Purkinje cell, was next analyzed for its survival in *trkB* and *trkC* single and double mutants. In contrast to cerebellar granule neurons of the EGL, no significant numbers of pyknotic Purkinje cells were observed in either *trkB*^{-/-}; *trkC*^{+/-} or *trkB*^{+/-};*trkC*^{-/-} mice (data not shown). Moreover, the total number of Purkinje cells was unchanged in *trkB/trkC* double mutant mice compared with the single mutant and wild-type mice (data not shown). Instead, sections stained for calbindin revealed a marked decrease in the highly branched dendritic trees of Purkinje cells in mice carrying mutant

trkB and *trkC* alleles (Fig. 7). The thickness of dendritic trees was reduced by 50% and 48% in *trkB*^{-/-}; *trkC*^{+/-} and *trkB*^{+/-};*trkC*^{-/-} mice, respectively (Table 2). Both *trkB*^{-/-} and *trkC*^{-/-} single mutants showed mild, yet significant reductions in dendritic arborizations. These results indicate that Purkinje cell survival is much less sensitive to reduced levels of *TrkB* and *TrkC* compared with granule cell survival. Purkinje cell survival may therefore depend on trophic signals distinct from neurotrophins. In contrast, Purkinje cell dendritic differentiation directly or indirectly depends on cooperative signaling via *TrkB* and *TrkC*.

Discussion

This study provides genetic evidence for a physiological role of endogenous neurotrophins in the survival of two

Figure 5. Premigratory cerebellar granule cells depend on *TrkB* and *TrkC* for survival. Percentage of pyknotic cells (means $\pm$ S.E.) in the EGL in cresyl violet-stained mid-sagittal sections of P12 mice. The data were obtained from three mice of all genotypes. For abbreviations, see Fig. 2. Statistical analysis was carried out using Student's *t*-test and is indicated where significant. (*) *P* < 0.01; (**) *P* < 0.001.

Figure 6. Increase in the number of TUNEL-positive cerebellar granule cells in mice carrying mutant *trkB* and *trkC* alleles. Mid-sagittal section of cerebella from P12 wild-type (A), *trkB*^{-/-} (B), *trkB*^{+/-};*trkC*^{-/-} (C), and *trkB*^{-/-}; *trkC*^{+/-} (D) stained by the TUNEL technique. Note the massive increase in tunel-positive cells in the external granule layer of mice with mutant *trkB* and *trkC* alleles. Magnification, 1000 $\times$.

Figure 7. Cerebellar Purkinje cells survive normally, but have reduced dendritic trees in mice carrying mutant *trkB* and *trkC* alleles. Mid-sagittal sections from P12 wild-type (A), *trkB*^{-/-}; *trkC*^{+/-} (C,G), *trkB*^{-/-} (E), *trkC*^{-/-} (F), and *trkB*^{+/-}; *trkC*^{-/-} mice (H) immunostained with a calbindin-specific antibody. Note the significant reduction of dendritic arborizations in two different *trkB*^{-/-}; *trkC*^{+/-} (C,G) and one *trkB*^{+/-}; *trkC*^{-/-} mutant (H) compared with the single mutants. To visualize the increase in the number of granule cells in the EGL of *trkB*^{-/-}; *trkC*^{+/-} compared with wild-type mice, cresyl violet-stained sections were included (D and B, respectively). (egl) External granule layer; (igl) internal granule layer; (ml) molecular layer; (pc) purkinje cell layer. Magnification, 200 $\times$.

populations of central neurons, namely, hippocampal and cerebellar granule cells. Moreover, TrkB and TrkC receptors, which are coexpressed in these neurons (Klein et al. 1990; Bengzon et al. 1993; Lamballe et al. 1994; Tessarollo et al. 1993; Lindvall et al. 1994; Segal et al. 1995), act in a redundant fashion, indicating that activation of either of the two receptors is sufficient for survival. The dramatic increase in cell death in P12 *trkB*^{-/-}; *trkC*^{+/-} and *trkB*^{+/-}; *trkC*^{-/-} mice is remarkable considering the fact that these cells still carry one functional allele of either the *trkB* or *trkC* gene. Similar effects of Trk receptor dosage have been

observed recently in vitro during the analysis of the survival-promoting effects of NT3 on primary sensory neurons derived from crosses of double heterozygous *trk* mutant mice (Davies et al. 1995). Hippocampal and cerebellar granule neurons are the first examples of cells in which two different neurotrophin receptors act in a directly compensating, redundant fashion. Thus, these neurons can survive in vivo in the presence of either the TrkB ligands (BDNF and NT4/5) or the TrkC ligand (NT3), but have no other source of survival factors. Other CNS neuron, such as motoneurons, which respond very well to exogenous neurotrophins (Oppenheim et al. 1992; Sendtner et al. 1992; Yan et al. 1992; Henderson et al. 1993), survive even in the absence of both TrkB and TrkC receptors (L. Minichiello, F. Piehl, and R. Klein, unpubl.), indicating that they can use other trophic factors distinct from neurotrophins.

Our findings in the CNS are in sharp contrast to most peripheral neurons, which critically depend on a single neurotrophin/Trk receptor signaling pathway. Hence, the effects on peripheral neuron survival of combined deficiencies in either Trk receptors (Minichiello et al. 1995) or neurotrophins (Ernfors et al. 1995) are generally additive and indicate that neurotrophins act in a largely noncompensatory and nonredundant fashion. This suggests that the survival of most peripheral neurons may follow different mechanisms than central neurons. For instance, the survival of peripheral sensory neurons of the vestibular ganglion is regulated by limiting amounts of the TrkB ligand BDNF, as demonstrated in mice heterozygous for a BDNF null allele (Bianchi et al. 1996). In the CNS, it remains to be determined whether or not the numbers of granule neurons are also regulated by limiting amounts of TrkB and TrkC ligands. It is hoped that mice triple heterozygous for BDNF, NT3, and NT4/5 null alleles will shed light on this issue.

Some populations of peripheral neurons behave in exactly the opposite way to hippocampal and cerebellar granule neurons, in that they appear to require two different neurotrophin/Trk signaling pathways for survival during development. For instance, essentially all sympathetic neurons require NGF/TrkA, and ~50% also require NT3 for survival (Crowley et al. 1994; Farinas et al. 1994; Smeyne et al. 1994). Likewise, a fraction of vestibular neurons requires TrkC, but the same reduction of vestibular neurons is observed in *trkB*^{-/-} and *trkB*^{-/-}; *trkC*^{-/-} mutant mice, indicating that the TrkC-dependent population was also affected in the *trkB* single mutant mice (Minichiello et al. 1995). Whether or not this reflects a simultaneous dependence or a sequential requirement because of a switch in neurotrophin dependence (Davies 1994b) remains to be determined. In any case, direct compensation between two Trk receptors has not been observed in any peripheral neuron population.

Our results provide genetic support for current hypotheses on neurotrophin function in the hippocampus. Most neurotrophins and the receptors TrkB and TrkC are coexpressed in the dentate gyrus and their expression is markedly increased as a result of brain insults, suggest-

Table 2. *Reduced dendritic differentiation of cerebellar Purkinje cells in *trkB/trkC* double mutant mice*

Genotype	No. of animals	Length of dendritic tree $\pm$ S.E.M. (μ m)	Reduction compared with wild-type (%)	Reduction compared with single mutants (%)
Wild-type	4	143 $\pm$ 6.5		
<i>trkB</i> $-/-$	4	111 $\pm$ 6.5	22 ^a	
<i>trkC</i> $-/-$	4	115 $\pm$ 8	19 ^a	
<i>trkB</i> $-/-$; <i>trkC</i> $+/-$	5	72 $\pm$ 6.5	50 ^b	36 ^b
<i>trkB</i> $+/-$; <i>trkC</i> $-/-$	6	75 $\pm$ 2.5	48 ^b	35 ^b

Measurements on the length of dendritic trees of Purkinje cells were performed on calbindin-stained mid-sagittal sections of P12 mice (see Fig. 7). The mean length of dendritic trees, expressed in micrometers, was determined by measuring the thickness of the molecular layer on both sides of each of three different lobes per cerebellum on the above-indicated number of animals. Statistical analysis was carried out using Student's *t*-test.

^a*P* < 0.01.

^b*P* < 0.001.

ing, together with other findings, that neurotrophin signaling may protect against neuronal damage by inducing sprouting and synaptic reorganization (Lindvall et al. 1994; Isackson 1995). The fact that TrkB and TrkC mediate a physiological survival signal makes it very likely that they also have a similar role in pathophysiological conditions that threaten neuronal viability. Hippocampal pyramidal neurons have been shown in vitro to survive independently of the ligands of TrkB and TrkC receptors (Collazo et al. 1992; Ip et al. 1993; Ohsawa et al. 1993). In *trkB/trkC* double mutants, no significant apoptosis was detected in the Ammon's horn, where hippocampal pyramidal neuron cell bodies are located. In contrast, the survival of dentate gyrus neurons was enhanced by BDNF and NT3 in vitro (Lowenstein and Arsenault 1996), consistent with the observed selective vulnerability of dentate gyrus neurons in *trkB* $-/-$; *trkC* $+/-$ and *trkB* $+/-$; *trkC* $-/-$ mutant mice.

The kinetic analysis of cell death in *trkB/trkC* double mutants revealed that dentate gyrus neurons require TrkB and TrkC receptors shortly after the period of naturally occurring cell death. TrkB and TrkC therefore do not appear to have a role during the initial proliferative phase, as we have shown recently for TrkB in the trigeminal system (Piñon et al. 1996). In this respect, dentate gyrus neurons behave more similarly to sensory neurons of the vestibular ganglion, which require TrkB receptors for survival after their axons have reached the target organ (Schimmang et al. 1995). Therefore, rather than supporting the survival of proliferating neuronal precursors, TrkB and TrkC appear to be mainly required to support postmitotic dentate granule cells and their excitatory synaptic connections within the hippocampus.

We found an inverse correlation between the number of apoptotic cells in the dentate gyrus and the cross-sectional area of calbindin-positive mossy fibers, suggesting that the number of functional granule cells was also reduced in *trkB/trkC* double mutant mice. In addition to a reduction in cross-sectional area of mossy fibers, the intensity of calbindin staining appeared reduced in most double mutants (not shown) consistent with earlier reports on BDNF $-/-$ mice (Jones et al. 1994) and

with enhanced calbindin expression in neurotrophin treated cultures (Collazo et al. 1992; Ip et al. 1993). Whether or not calbindin has a physiological role in mediating the survival function of neurotrophins, possibly by stabilizing Ca²⁺ homeostasis (Lindvall et al. 1994), remains to be determined. Also, because of the limited life span of the animals analyzed above, we do not know whether eventually all dentate gyrus neurons would undergo cell death, or whether only a calbindin-positive subpopulation is affected.

A remarkably similar picture was observed in the cerebellum. Like dentate gyrus granule neurons, cerebellar granule neurons coexpress TrkB and TrkC, but not TrkA receptors, and respond to exogenous neurotrophins in culture, albeit to varying degrees. Whereas BDNF and NT4/5 promote the survival of cultured granule neurons, NT3 appears to induce more subtle responses including induction of c-Fos and change in neurite outgrowth (Segal et al. 1992, 1995; Leingärtner et al. 1994; Gao et al. 1995). The fact that both *trkB* $+/-$; *trkC* $-/-$ and *trkB* $-/-$; *trkC* $+/-$ mice show marked increases in numbers of apoptotic granule neurons suggests that the endogenous ligands of TrkB and TrkC are equally important for the survival of postnatal cerebellar granule neurons. Interestingly, cerebellar granule neurons appear to require TrkB and TrkC mediated signaling before descending from the EGL to the IGL. These findings are consistent with previous observations in organotypic cultures, where BDNF induced c-Fos in the EGL, but not in the IGL or Purkinje cell layer (Segal et al. 1992). Moreover, TrkB expression was shown to be down-regulated in IGL neurons as compared with migrating granule cells (Gao et al. 1995). Together, these results suggest that cerebellar granule neurons, once fully matured and situated in the IGL, become independent of endogenous neurotrophins presumably by up-regulating receptors for other trophic factors.

In contrast to granule neurons, the survival of Purkinje cells was not affected in *trkB* $-/-$; *trkC* $+/-$ and *trkB* $+/-$; *trkC* $-/-$ mice. This suggests that either the critical levels of TrkB/C signaling are much lower for Purkinje cells, or that endogenous neurotrophins do not

promote Purkinje cell survival. Instead, the maturation of Purkinje cell dendrites was markedly reduced in *trkB*^{-/-};*trkC*^{+/-} and *trkB*^{+/-};*trkC*^{-/-} mice compared with wild-type and single mutants. It is known that Purkinje cell dendritic differentiation is regulated to a major extent by contacts with the parallel fibers of granule neurons (Altman 1982; Baptista et al. 1994), rather than by intrinsic factors. Considering the degree of cell death in cerebellar granule neurons, it is possible that the observed reduction in dendritic arborization is indirectly a result of reduced interactions between granule and Purkinje cells. Alternatively, neurotrophins may have a direct role in promoting Purkinje cell differentiation. NT3 produced by cerebellar granule neurons has been shown to induce neurite sprouting of cultured Purkinje cells (Lindholm et al. 1993), implicating NT3 as one of the factors promoting Purkinje cell differentiation.

In summary, we have shown that, contrary to current belief, neurotrophins are important for the survival of at least two major CNS neuron populations. Whether or not other CNS neurons depend on endogenous neurotrophins for survival will require new longer-lived mouse models in which the expression of Trk receptors can be regulated tissue-specifically and/or temporally.

Materials and methods

Mouse mutants

The generation and genotypic analysis of *trkA*, *trkB*, and *trkC* mutant mice has been described previously (Klein et al. 1993, 1994; Smeyne et al. 1994; Schimmang et al. 1995). The phenotypes of double mutants were analyzed in a mixed 129xC57Bl/6 background.

Histology

Double heterozygous mutant mice were crossed and their litters fixed by cardiac perfusion at the indicated time points with 4% paraformaldehyde in 0.1 M phosphate buffer. Prior to fixation, tail biopsies were taken for genotypic analysis. Brains were dissected, postfixed for 24 hr, left in sucrose until they sank to the bottom of the tube, mounted in tissue freezing medium (Tissue-Tek), and then frozen on dry ice.

Hippocampus

Serial cryosections were taken at a thickness of 40 μ m and further processed as described (Minichiello et al. 1995) in order to do free-floating immunohistochemistry and Nissl and TUNEL staining on the same animals. For quantitative analysis, Nissl-stained hemisections were selected from three areas, rostral, middle, and temporal dentate gyrus (Gould et al. 1991), and the numbers of pyknotic cells counted at 1000 $\times$ magnification in the supra- and infrapyramidal blades of the granule cell layer. The hilus of the dentate gyrus was excluded from this analysis, as it contains other cell types than granule cells. Because the numbers of pyknotic cells of the three levels of the dentate gyrus were not significantly different within the same animal (data not shown), the total number of pyknotic cells per dentate gyrus was estimated by multiplying the average cell count by the number of sections between the most rostral and

the most temporal sections. Because of the smaller size of mutant brains, the average number of 40- μ m sections was 37 and 27, for wild-type and mutant P12 brains, respectively. The total number of dentate gyrus neurons was found to be reduced by 15–25% in P12 mutants compared with wild-type mice (data not shown). Because this correlated well with the ~20–25% smaller brain size of mutant mice, the numbers of pyknotic cells were reported without correcting for the total number of dentate gyrus neurons.

Cerebellum

Serial cryosections were taken at a thickness of 8 μ m and cresyl violet Nissl-stained mid-sagittal sections were selected for analysis. The percentage of pyknotic cells in the EGL was determined on ~2000 healthy cells ($n=3$ for each genotype) counted in three different cerebellar lobes (lobes IV, V, and VI), although similar numbers of pyknotic cells were present in all cerebellar lobes. The length of the dendritic tree of Purkinje cells was determined (using an ocular micrometer) by measuring the thickness of the molecular layer on both sides each of three different lobes per cerebellum on two adjacent mid-sagittal sections stained with calbindin (see below) on four to six animals per genotype.

TUNEL staining

Forty-micrometer coronal floating sections were postfixed with 4% paraformaldehyde, endogenous peroxidase was blocked with 2% H₂O₂, and the tissue was permeabilized in 0.3% triton X-100, 0.1% sodium citrate. TUNEL staining was performed according to manufacturer's specifications (Boehringer). After incubation with a secondary biotinylated antibody and the ABC reagent, peroxidase was reacted with 0.05% diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide.

Calbindin immunostaining

Forty-micrometer coronal sections were pretreated as described (Minichiello et al. 1995). Goat anticalbindin antibodies (Sigma) were incubated (1:400 dilution) in Tris-buffered saline, containing 0.5% Triton X-100 and 1% normal goat serum. Staining with the ABC method was as described in legend of Figure 2.

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