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# Cortical Degeneration in the Absence of Neurotrophin Signaling: Dendritic Retraction and Neuronal Loss after Removal of the Receptor TrkB

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## Summary

**To examine functions of TrkB in the adult CNS, TrkB has been removed from neurons expressing CaMKII, primarily pyramidal neurons, using Cre-mediated recombination. A floxed *trkB* allele was designed so that neurons lacking TrkB express tau-β-galactosidase. Following *trkB* deletion in pyramidal cells, their dendritic arbors are altered, and cortical layers II/III and V are compressed, after which there is an apparent loss of mutant neurons expressing the transcription factor SCIP but not of those expressing Otx-1. Loss of neurons expressing SCIP requires deletion of *trkB* within affected neurons; reduction of neuronal ER81 expression does not, suggesting both direct and indirect effects of TrkB loss. Thus, TrkB is required for the maintenance of specific populations of cells in the adult neocortex.**

## Introduction

Neurotrophins have been shown to promote neuronal survival of a variety of neuronal populations in both the peripheral and central nervous systems (Reichardt and Fariñas, 1997). Neurotrophins are a family of highly related, small, secreted proteins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). They exert their effects on neurons mainly through Trk receptor tyrosine kinases. NGF specifically activates TrkA, BDNF and NT-4/5 activate TrkB, and NT-3 activates TrkC and, to a lesser degree, TrkA and TrkB. Analyses of mice with mutations in genes for neurotrophins or their receptors have demonstrated that neurotrophins are required for survival of distinct populations of neurons in the PNS during embryogenesis (reviewed in Reichardt and Fariñas, 1997). Very few defects, however, have been detected in the CNS of these mouse mutants. Among these are deficits in differentiation of interneurons and impaired maturation of Purkinje cell dendritic trees in the *BDNF* mutant (Jones et al., 1994; Schwartz et al., 1997), and small increases in hippocampal and cerebellar granule cell apoptosis in the *trkB* mutant (Minichiello and Klein, 1996; Alcántara et al., 1997).

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The *trkB* gene is widely expressed in the developing and adult brain. Almost every anatomical structure in the adult brain is positive for TrkB immunoreactivity (Yan et al., 1997). One of the TrkB ligands, BDNF, can support survival of many types of CNS neurons in vitro, including cortical neurons, hippocampal neurons, nigral dopaminergic neurons, basal forebrain cholinergic neurons, and motor neurons (reviewed by Korsching, 1993; Ghosh et al., 1994). The *trkB* null mutants die during first 3 postnatal weeks due to severe sensory deficits. As brain development is not completed during their lifespan, this has made it difficult to determine the requirements for these molecules during the entire span of CNS development and may explain why major CNS abnormalities have not been seen in mutant animals. Besides the traditional role in neuronal survival, TrkB signaling has been implicated in many other aspects of neuronal development and in regulation of a neuronal circuitry (reviewed by Cellerino and Maffei, 1996; Reichardt and Fariñas, 1997; McAllister et al., 1999). These include axonal sprouting; dendritic growth of cortical pyramidal cells; maturation of the visual cortex, including ocular dominance column formation (e.g., Cabelli et al., 1997); and synaptic transmission and long-term potentiation in the hippocampus. To examine the roles of TrkB in the maintenance and function of the adult nervous system, we have used the bacteriophage Cre/loxP recombination system to generate a mouse strain in which *trkB* is deleted in mature pyramidal neurons in the neocortex and CA1 region of the hippocampus by expression of *cre* directed by the promoter for the α subunit of $Ca^{2+}$/calmodulin-dependent protein kinase II (αCaMKII). Here, we report that in the neocortex of the *trkB* conditional mutant, dramatic changes in dendritic structure are accompanied by compression of the neocortex, which is followed by severe neuronal loss. Thus, the TrkB receptor is necessary for maintenance of the integrity of the CNS, and signaling deficits result in degenerative changes similar to those observed in certain diseases.

## Results

### Generation of αCaMKII Promoter-Directed *trkB* Mutant

To study the roles of TrkB in the adult brain, we have generated a floxed (flanked by two loxP sites) *trkB* allele termed *fBZ*, in which a floxed *trkB* cDNA and a following reporter gene, *tau-lacZ*, are inserted into the first coding exon of the wild-type *trkB* allele (Figure 1; B. Xu et al., submitted). Unlike the wild-type *trkB* allele, which encodes both a full-length TrkB receptor tyrosine kinase and a truncated TrkB receptor lacking the kinase domain (Klein et al., 1990), the *fBZ* allele only expresses the full-length receptor at ~25% of the normal amount throughout the brain. After the floxed *trkB* cDNA is deleted by Cre-mediated recombination, the *fBZ* allele becomes a $trkB^{lacZ}$ allele, and the *tau-lacZ* gene is expressed in cells that previously expressed TrkB (Figure 1). The *cre* transgenic mouse used in this study contains a *cre*

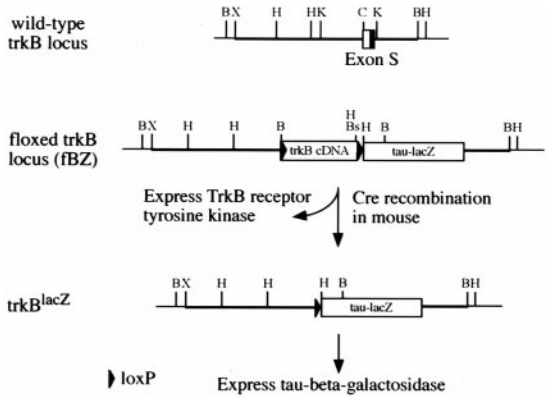


Figure 1. Schematic Diagram of the Floxed *trkB* Allele

(Top) Wild-type *trkB* locus. Exon S is the first coding exon of the *trkB* gene and encodes the signal peptide of TrkB.

(Middle) The *fBZ* locus. The *fBZ* locus was created by replacing most of Exon S with a floxed *trkB* cDNA and a *tau-lacZ* reporter gene. Both the *trkB* cDNA unit and the *tau-lacZ* gene have their own polyadenylation signal sequences.

(Bottom) The $trkB^{lacZ}$ locus. When the floxed *trkB* cDNA unit is deleted by Cre recombinase, a $trkB^{lacZ}$ locus is generated. Expression of tau-β-galactosidase is under the control of the *trkB* promoter.

Abbreviations: B, BamHI; Bs, multiple BamHI sites; C, ClaI; H, HindIII; K, KpnI; and X, XbaI.

transgene under the control of the αCaMKII promoter, termed *CaMKcre* (B. Xu et al., submitted). Mice heterozygous for *fBZ* were crossed with *CaMKcre* transgenic mice to generate mice heterozygous for both *fBZ* and *CaMKcre* (*fBZ/+;CaMKcre/+*). The *trkB* conditional knockouts (*fBZ/fBZ;CaMKcre/+*) were generated from crosses between mice heterozygous for the *fBZ* allele (*fBZ/+*) and mice heterozygous for both *fBZ* and *CaMKcre*. The conditional mutants can survive at least 3 months.

To identify where the *fBZ* allele in the brain is deleted after expression of the *CaMKcre* transgene, we examined expression of the *tau-lacZ* gene in mice heterozygous for both *fBZ* and *CaMKcre* (Figure 2). Since the *tau-lacZ* gene is under the control of the *trkB* promoter, it is expressed only in TrkB-expressing neurons in which the floxed *trkB* cDNA has been deleted by Cre-mediated recombination. Tau-β-galactosidase is expressed in the neocortex, hippocampus, and substantia nigra (Figures 2C and 2D). Some cells in the striatum, amygdala, and basal forebrain septal nuclei were also positive for β-galactosidase (Figure 2C; data not shown). The blue staining in the thalamus and the corpus callosum was due to localization of tau-β-galactosidase in the axons of cortical neurons (Figures 2C and 2D). When a heterozygous *fBZ/+* mouse does not contain the *CaMKcre* transgene, no tau-β-galactosidase is expressed (Figure 2A). Expression of the tau-β-galactosidase reporter was detected as early as postnatal day 14 (P14), but only in a small number of neurons localized primarily to layers II/III and V (Figure 2B). In the neocortex, maximal expression was seen at P29 and later ages (Figure 2C). Counts of β-galactosidase-immunoreactive neurons were similar at P28 and P47 (65 ± 5 at P28 versus 61 ± 5 at P47 for a 190 μm wide coronal stripe). Therefore, deletion of the floxed trkB locus appears to plateau at ~P28. When the floxed *trkB* cDNA is deleted in all cells by Cre-mediated recombination, no TrkB proteins are expressed in the brain (Figure 2E).

Cortical neurons include excitatory pyramidal cells and inhibitory GABAergic interneurons. To determine whether the floxed *trkB* is preferentially deleted in specific types of cortical neurons by expression of *cre* driven by the *αCaMKII* promoter, we performed double immunofluorescence staining for β-galactosidase and two $Ca^{2+}$-binding proteins, calbindin and parvalbumin, that mark two nonoverlapping populations of cortical interneurons (Hendry et al., 1989). As shown in confocal images in Figures 3A–3L, most β-galactosidase-positive neurons do not express the two $Ca^{2+}$-binding proteins in the neocortex of *fBZ/+;CaMKcre/+* mice. A small number of neurons expressing both β-galactosidase and calbindin could be identified in layers II/III, where many neurons express calbindin (Figures 3G–3I). These results indicate that most of the targeted neurons in the *CaMKcre*-mediated *trkB* conditional mutant are pyramidal neurons. Indeed, all tau-β-galactosidase-expressing neurons in layer V also express αCaMKII, a marker for pyramidal neurons (Figures 3M–3O).

The *CaMKcre*-mediated deletion of the floxed *trkB* cDNA should reduce expression of the TrkB receptor in the *trkB* conditional mutant. To determine the overall loss of TrkB protein, a protein extract was prepared from the dissected posterior halves of the neocortex of 6-week-old mice, and an affinity-purified antibody to the TrkB extracellular domain was used to quantitate TrkB protein in a Western blot. Compared with an *fBZ/fBZ* mouse, the level of TrkB is reduced by 47% ± 14% (n = 4) in the *trkB* conditional mutant (Figure 2F).

### The Neocortex Is Compressed in the *trkB* Conditional Mutants

The gross anatomical structure of the neocortex was examined on sagittal sections of both 6-week-old and 10-week-old brains after Nissl staining, and similar phenotypes were observed at both ages. As illustrated in Figure 4, where sections of 10-week-old brain are shown, no significant differences in the cytoarchitectonic structure of the visual cortex were detected among animals with genotypes of *fBZ/+*, *fBZ/+;CaMKcre/+*, or *fBZ/fBZ*. This result suggests that the reduced level of TrkB expression in the *fBZ/fBZ* mouse does not cause significant abnormalities in the neocortex. The results also indicate that the expression of tau-β-galactosidase in the *fBZ/+;CaMKcre/+* mouse is not toxic to cortical neurons. However, the visual cortex of the conditional mutant is severely compressed, especially layers II/III and layer V (Figure 4). A similar compression occurs in the somatosensory cortex (data not shown). These results show that removal of the TrkB receptor from a subpopulation of cortical pyramidal neurons results in collapse of several layers in the neocortex.

### Altered Morphologies of *trkB* Mutant Neurons

The fates of targeted neurons were monitored by following the expression of tau-β-galactosidase with X-gal staining or anti-β-galactosidase immunostaining in brain sections from animals of various ages. Two genotypes of mice were used in the analyses. In the *fBZ/+;*

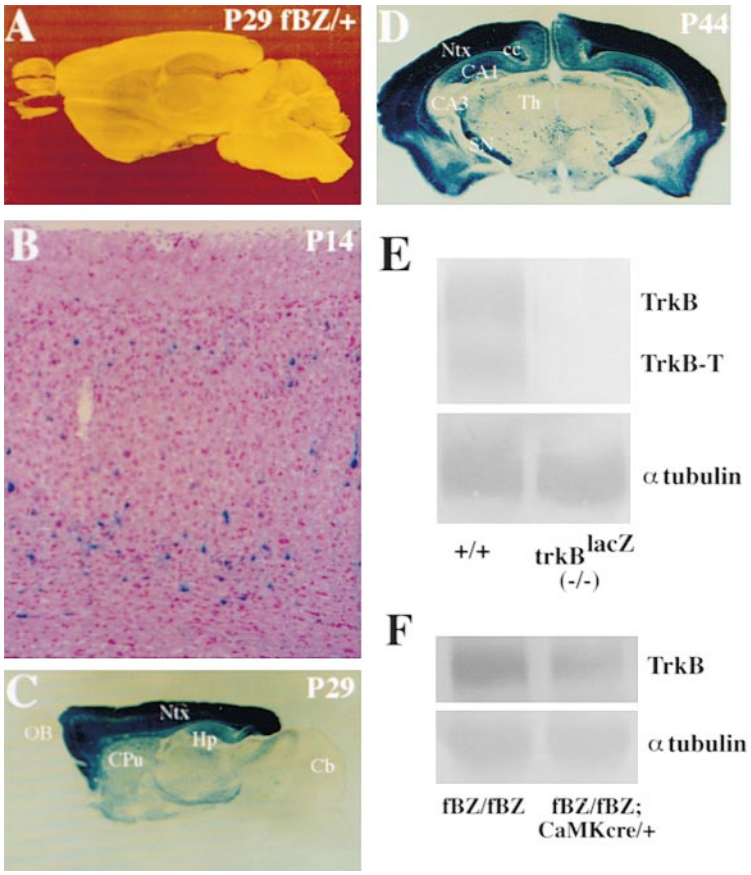


Figure 2. Pattern of *CaMKcre*-Mediated Deletion of the Floxed *trkB* Allele in the Brain

(A–D) Expression of the reporter gene *tau-lacZ* in the brain. Expression of tau-β-galactosidase was determined by X-gal staining.

(A) The *tau-lacZ* gene was not expressed in mice heterozygous for the *fBZ* allele (no Cre expression).

(B) A small number of neurons in the neocortex are positive for X-gal staining at P14. These are primarily located in layers II/III and V.

(C and D) Representative sagittal (C) and coronal (D) sections from P29 and P44 mice heterozygous for both the *fBZ* allele and the *CaMKcre* transgene show expression of the *tau-lacZ* gene in the neocortex (Ntx), hippocampus (Hp), substantia nigra (SN), and caudate-putamen (CPu). Animal ages (days) are indicated in the figure. Abbreviations: Cb, cerebellum; cc, corpus callosum; OB, olfactory bulb; and Th, thalmus.

(E) TrkB expression in the $trkB^{lacZ}$ allele. Protein extracts were prepared from P0 forebrains. The blot was probed with antibodies to TrkB and α-tubulin sequentially. Abbreviations: TrkB, full-length TrkB receptor, and TrkB-T, truncated TrkB proteins.

(F) Western blot analyses of TrkB protein. Protein extracts were prepared from the posterior halves of the neocortices of 6-week-old mice. The blot was probed with antibodies to TrkB and α-tubulin sequentially. Mouse genotypes are indicated. Blots were quantitated on a Fuji Multiimager. Expression of Cre results in loss of 47 ± 14% (n = 4) of TrkB kinase protein.

*CaMKcre/+* control mouse, neurons positive for β-galactosidase still have one wild-type *trkB* locus and should survive normally. In the *fBZ/fBZ;CaMKcre/+* conditional mutant, neurons expressing tau-β-galactosidase are $trkB^{-/-}$ mutant cells. Antibodies to β-galactosidase stained cell bodies and apical dendrites of neocortical neurons in 6-week-old control mice (Figures 5A and 5C). Basal dendrites and axons are also stained in some tau-β-galactosidase-containing neurons. While there are differences in the density of tau-β-galactosidase-containing neurons between layers, these cells can be found in every layer from layer II to layer VI (Figure 5A). The general staining over the whole section must reflect localization of tau-β-galactosidase in axons and dendrites and not background, because no staining was observed in nuclei (Figure 5A) or on brain sections from *fBZ/fBZ* mice lacking the *CaMKcre* transgene (data not shown; see also Figure 2A). Interestingly, in *trkB* conditional mutants at the same age, most neurons expressing tau-β-galactosidase have strong expression in their cell bodies, but dramatically reduced staining in their dendritic processes (Figures 5B and 5D). This reduction in dendritic staining was observed in all three pairs of 6-week-old mice we analyzed. Reduction of staining in the processes of *trkB* mutant neurons can be detected as early as 4 weeks in mutant mice (Figure 5E and 5F). However, counts of tau-β-galactosidase-expressing neurons are not significantly different in the visual cortex between control and conditional mutant mice, even at 6 weeks (Figure 5G). These results indicate that there are alterations of neuronal morphology in the *trkB*-targeted neurons that occur well before possible neuronal death.

To directly examine the morphologies of *trkB* mutant neurons, we injected biocytin, along with NMDA, into the visual cortex of the left hemisphere of anesthetized animals. Coinjection of NMDA has been shown to enhance the uptake of biocytin by neurons (Jiang et al., 1993). One day after injection, animals were fixed by perfusion, and their brains were processed for fluorescent immunohistochemistry for β-galactosidase and biocytin. Dendritic structures of neurons labeled by biocytin that also expressed tau-β-galactosidase in the *fBZ/fBZ;CaMKcre/+* conditional mutant were reconstructed and compared with those in the *fBZ/+; CaMKcre/+* control animal. Our comparison focused on pyramidal neurons in layers II/III, since they were the majority of neurons labeled by biocytin using our procedure. In comparison with $trkB^{+/-}$ neurons (Figures 6A and 6B), $trkB^{-/-}$ neurons have thinner dendrites, especially apical dendrites (Figures 6C and 6D). The average diameter of the apical dendrite of a $trkB^{-/-}$ neuron is reduced by 41% in comparison with that of a $trkB^{+/-}$ neuron (Figure 6E). Moreover, Sholl analyses indicate that the average dendritic complexity of $trkB^{-/-}$ neurons is reduced (Figure 6F). The reduced dendritic complexity is due to fewer and/or shorter branches from primary dendrites, since the average number of primary dendrites in $trkB^{-/-}$ neurons is similar to that found in control neurons (Figure

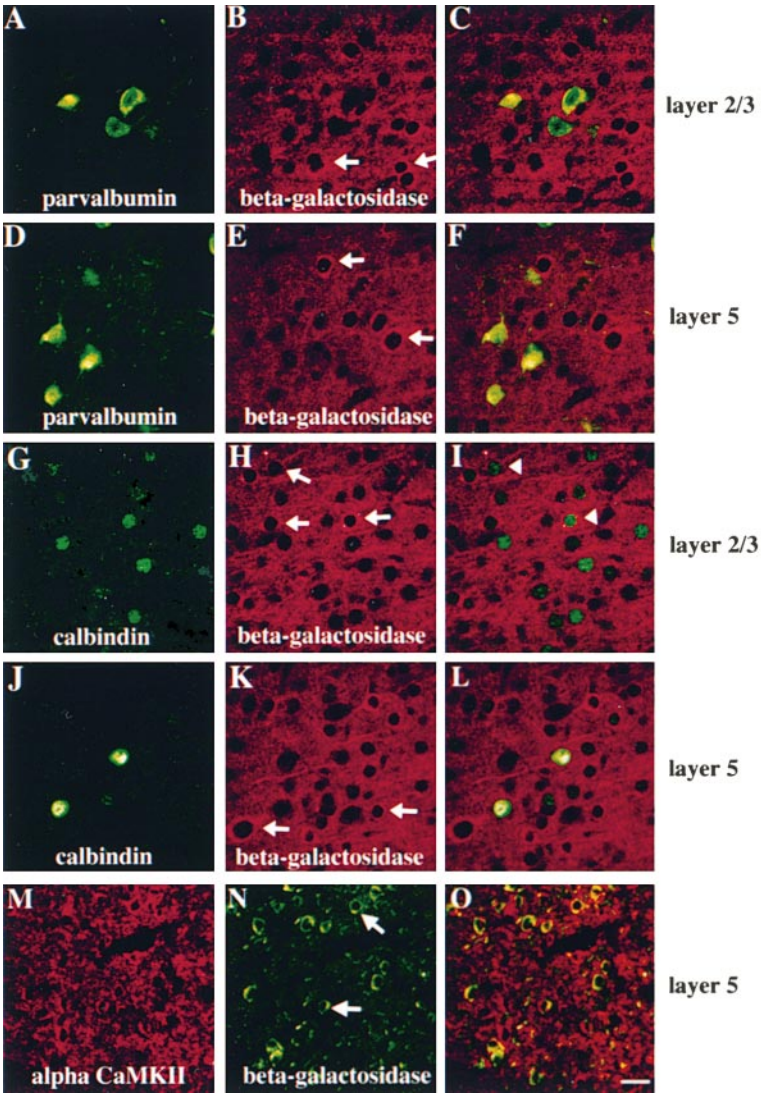


Figure 3. Targeting of the Floxed *trkB* Allele in Pyramidal Neurons by the *CaMKcre* Transgene in the Neocortex

(A–F) Absence of coexpression of parvalbumin and tau-β-galactosidase in the P65 cortex. Note absence of colabeled neurons in both layer II/III and layer V of the visual cortex (C and F). Some of tau-β-galactosidase-positive neurons are denoted by arrows in (B) and (E).

(G–L) Infrequent coexpression of calbindin and tau-β-galactosidase in the P65 cortex. Two colabeled neurons in layer II/III of the visual cortex are indicated by arrowheads (I), but the majority of calbindin-expressing neurons do not express tau-β-galactosidase. Colabeled neurons are not observed in layer V of the visual cortex (L). Some of the neurons expressing tau-β-galactosidase are indicated by arrows in (H) and (K).

(M–O) Expression of tau-β-galactosidase is restricted to neurons coexpressing αCaMKII in the P44 cortex. Coexpressing neurons are yellow in (O). Note that not all neurons expressing αCaMKII express tau-β-galactosidase. Immunohistochemistry was performed on sagittal brain sections of *fBZ/+;CaMKcre/+* mice. Scale bar, 20 μm.

6F). Interestingly, cell bodies of *trkB*$^{-/-}$ neurons are smaller by 27% in the radial direction, although their width is only slightly reduced (Figure 6G). Therefore, cell bodies of *trkB*$^{-/-}$ null mutant neurons are smaller and more nearly round in shape. Some *trkB*$^{-/-}$ neurons have an oval shape, which rarely occurs in wild-type pyramidal neurons (Figure 6C). In summary, these results demonstrate that the TrkB receptor is essential for maintaining the normal morphologies of pyramidal neurons in the neocortex.

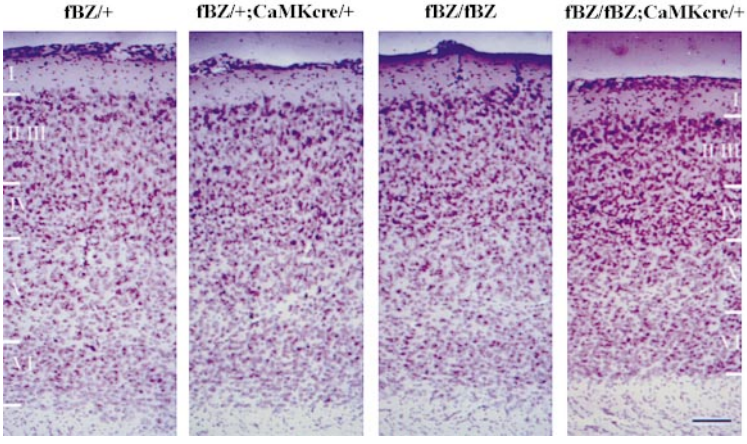


Figure 4. The Neocortex Is Compressed in the *trkB* Conditional Mutant at P72

Sagittal sections of the P72 visual cortex were stained with cresyl violet and compared. The neocortex in *fBZ/+*, *fBZ/+;CaMKcre/+*, or *fBZ/fBZ* mice does not appear to be compressed. Note that the neocortex in the *fBZ/fBZ;CaMKcre/+* conditional mutant is compressed, with shrinkage particularly obvious in layers II/III and V. Layers I, IV, and VI were not compressed significantly. Scale bar, 100 μm.

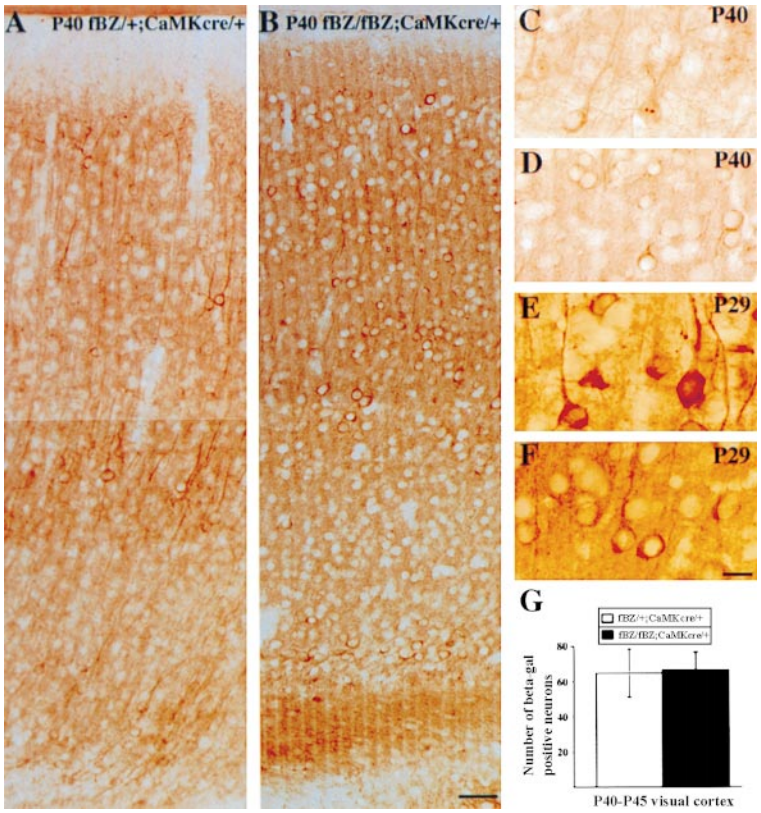


Figure 5. Reduction of Tau-β-Galactosidase Expression in the Dendrites of *trkB* Mutant Neurons

(A and B) Sagittal sections of control (A) and mutant (B) visual cortex of P40 mice. Note that most neurons do not express tau-β-galactosidase in their apical dendrites in the *fBZ/fBZ;CaMKcre/+* conditional mutant. Scale bar, 50 μm.

(C and D) Layer V neurons expressing tau-β-galactosidase in the visual cortex of control (C) or mutant (D) at P40. Staining in the apical dendrites is largely absent in *trkB* mutant neurons (D).

(E and F) Layer V neurons in the somatosensory cortex of a P29 *fBZ/+;CaMKcre/+* control mouse (E) or a P29 *trkB* conditional mutant (F). Staining in the apical dendrites is reduced in *trkB* mutant neurons (F).

(G) Counts of neurons expressing tau-β-galactosidase. Six 190 μm wide stripes of the visual cortex on sagittal sections from three P40- to P45-old animals of each genotype were counted. Data are presented as mean ± SD. The number of tau-β-galactosidase-expressing neurons was not reduced significantly in the cortex of animals in which *trkB* was deleted by *CaMKcre*. Two-tailed t test, $p = 0.80$.

Scale bar, 50 μm (A and B) and 20 μm (C–F).

**Many Cortical Neurons Require TrkB for Survival**

To determine whether TrkB activation is required for survival of cortical neurons, we analyzed older *trkB* conditional mutant mice. β-galactosidase immunohistochemistry of brain sections from 10-week-old and older animals showed that, compared to controls, there is a significant reduction in the number of tau-β-galactosidase-expressing neurons in the *trkB* conditional mutant (Figures 7A–7D). Neurons expressing tau-β-galactosidase were counted across all layers of the visual cortex in several stripes of sagittal sections. Results from four pairs of mice show that there is a 47% reduction in the number of tau-β-galactosidase-expressing neurons in the visual cortex of the *trkB* conditional mutant (Figure 7G). Interestingly, neither apical dendritic structure nor number of β-galactosidase-expressing neurons in the hippocampal CA1 region is apparently affected by loss of the TrkB receptor (Figures 7E and 7F). These results indicate that many neurons lacking TrkB may die in the neocortex, but not in the hippocampus, between 6 and 10 weeks of age.

Reduced numbers of tau-β-galactosidase-expressing neurons, however, could also result from downregulation of the *trkB* promoter in response to loss of the TrkB signaling. In an attempt to more directly show an increase in neuronal death in the neocortex, we performed TUNEL staining on brain sections of control and mutant mice at ages of 6, 8, and 10 weeks. No increase in the number of TUNEL-positive cells was detected in the *trkB* mutant (data not shown). An increase in the number of TUNEL-positive cells has also not been detected in the cortex of another independently generated trkB conditional mutant (Minichiello et al., 1999). The failure to detect increased death by TUNEL in the mutant could result from slow but progressive loss of cortical neurons over a period of weeks.

As another approach to confirm that many *trkB* mutant neurons are lost in 10-week-old and older *trkB* conditional mutant mice, effects of TrkB loss on several neuronal subpopulations were assessed using specific markers. The POU domain gene *SCIP* is expressed specifically by a subpopulation of neurons in layer V in both the developing and adult brain (Frantz et al., 1994a). In a P76 *fBZ/+;CaMKcre/+* mouse, the *fBZ* allele was deleted in ~58% (76 of 132) of SCIP-expressing neurons (Figures 8A–8C). Consistent with results from tau-β-galactosidase immunostaining (Figure 5), the number of SCIP-positive neurons in 6-week-old *trkB* mutants is not significantly different from that seen in control mice in both the visual cortex and the somatosensory cortex (Figure 8I). However, at 10 weeks, the number of SCIP-expressing neurons is significantly reduced in both cortical regions (Figures 8G–8I). If SCIP-expressing neurons die or lose SCIP expression directly because of loss of the TrkB receptor, those SCIP-postive neurons that express tau-β-galactosidase should be specifically affected. In fact, the percentage of SCIP-positive neurons that also express tau-β-galactosidase is reduced by 47%, from 58% in control (*fBZ/+;CaMKcre/+*) to 31% in the mutant (*fBZ/fBZ;CaMKcre/+*) visual cortex (Figures 8A–8F and 8J). Assuming that untargeted SCIP-expressing neurons are not affected, one expects a smaller percentage reduction ($0.47 \times 58\% = 27\%$) in the total population of SCIP-positive neurons in the mutant. Indeed, the observed reduction was ~19% in both the somatosensory cortex and the visual cortex (Figure 8I).

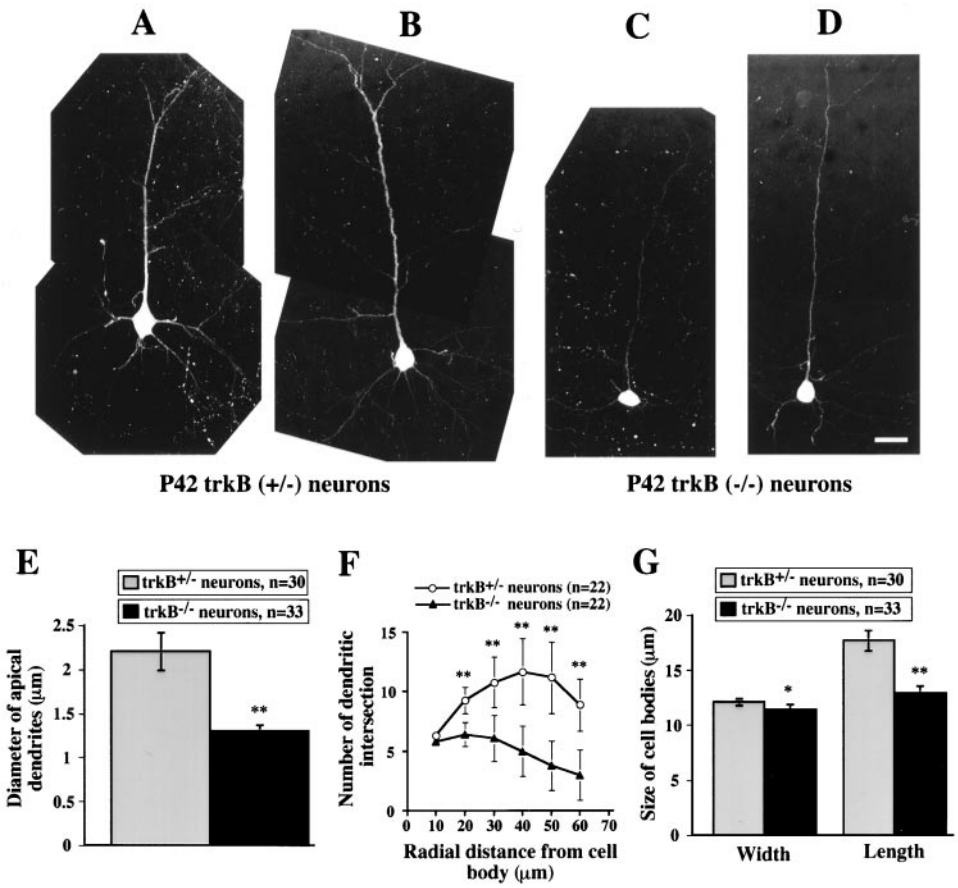


Figure 6. *trkB* Mutant Neurons in the Neocortex Have Thinner and Sparser Dendritic Arbors and Rounder Cell Soma

(A and B) Reconstructed *trkB*$^{+/-}$ neurons. The neurons were from layer II/III of the neocortex of a P42 *fBZ/+;CaMKcre/+* mouse and expressed tau-β-galactosidase.

(C and D) Reconstructed *trkB*$^{-/-}$ neurons. The neurons were from layer II/III of the neocortex of a P42 *fBZ/fBZ;CaMKcre/+* mutant mouse and expressed tau-β-galactosidase. Scale bar, 20 μm.

(E) Diameter of apical dendrites. Diameters were measured at locations ~4 μm from the soma. The average diameter of the dendrites in tau-β-galactosidase-expressing neurons lacking TrkB was reduced to ~50% of the diameter of similar dendrites from heterozygotes in which TrkB was not eliminated.

(F) Sholl analyses of dendritic structure. Absence of TrkB does not reduce the number of dentritic branches close to the cell soma but dramatically reduces this number at more distal locations.

(G) Shape of soma. Both length and width are maximum dimensions of a cell body. Length is significantly reduced by elimination of TrkB.

Data in (E) through (F) were collected from three pairs of mice and are presented as mean ± SEM. Two-tailed t test; asterisk, $p < 0.05$; double asterisk, $p < 0.01$.

If SCIP-expressing neurons were affected whether or not they expressed *tau-lacZ*, one would expect a much larger reduction in neuron number. For this reason, only those SCIP-expressing neurons that have been targeted appear to be affected in the *trkB* conditional mutant. Since counts of neither tau-β-galactosidase-expressing neurons (Figure 5) nor SCIP-expressing neurons (Figure 8) were detectably reduced in 6-week-old *trkB* conditional mutants, it seems unlikely that expression of both tau-β-galactosidase and SCIP are regulated by TrkB signaling. Therefore, these results suggest strongly that many *trkB*$^{-/-}$ neurons are lost in the neocortex between 6 and 10 weeks of age in the *trkB* conditional mutants. Thus, many pyramidal neurons in the neocortex appear to depend on TrkB for survival in adult animals.

Not all populations of neurons in the adult neocortex depend upon TrkB for survival. The homeodomain transcription factor Otx1 is expressed by many neurons in layers V and VI (Frantz et al., 1994b). Within layer V, neurons with axonal connections to subcortical targets (such as the colliculus and pons) localize Otx1 to their nuclei during early postnatal development; neurons with callosal connections do not express Otx1 (Figures 9D and 9E; Weimann et al., 1999). Although in the conditional mutant, the floxed *trkB* allele was targeted in 46% (11 of 24) of neurons expressing Otxl in their nuclei (Figures 9A–9C), the number of nuclear-Otx1-positive neurons in the visual cortex of 10-week-old *trkB* mutants was not significantly reduced below that present in the control animals (Figures 9D–9F). Thus, survival of neurons expressing nuclear Otx1 does not appear to depend on TrkB activation.

As shown in Figure 3, the *trkB* gene is not deleted in a subpopulation of interneurons that express parvalbumin. To determine whether these neurons are indirectly affected in the *CAMKcre*-directed *trkB* knockout,

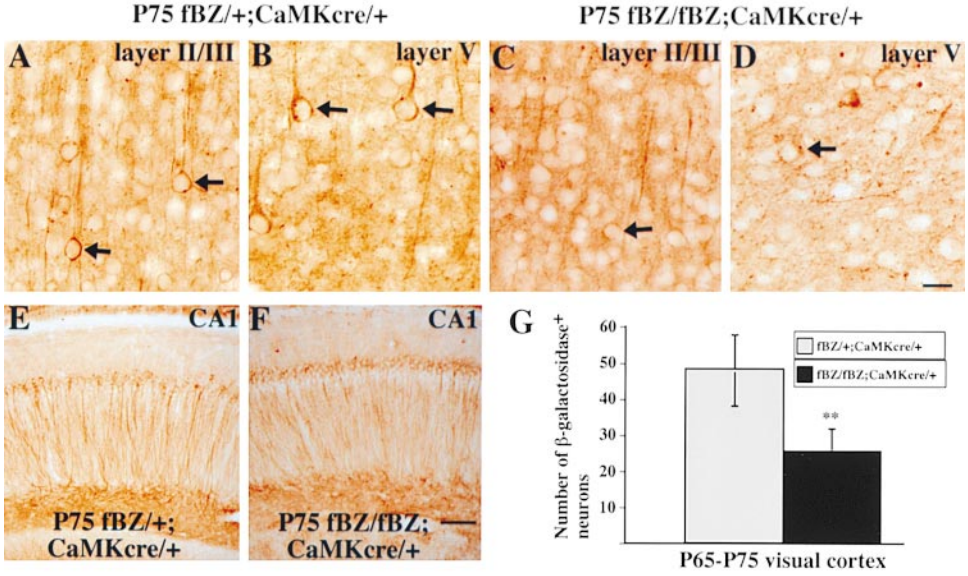


Figure 7. Number of Neurons Positive for Tau-β-Galactosidase Is Reduced in the Visual Cortex of P75 *trkB* Conditional Mutants

(A–F) Immunohistochemistry of β-galactosidase on sagittal brain sections of P75 *fBZ/+;CaMKcre/+* control (A, B, and E) or P75 *fBZ/fBZ; CaMKcre/+* mutant (C, D, and F) mice. Cortical layers II/III (A and C), layer V (B and D), and the hippocampal CA1 region (E and F) are shown. Examples of positive neurons are marked with arrows. Scale bar, 20 μm (A and D) and 100 μm (E and F).

(G) Counts of neurons expressing tau-β-galactosidase. Cell counts were obtained from eight 190 μm wide stripes of the visual cortex on sagittal sections from four pairs of *fBZ/+;CaMKcre/+* and *fBZ/fBZ;CaMKcre/+* brains with ages between P65 and P75. Note that absence of TrkB results in a 47% reduction in the number of tau-β-galactosidase-expressing neurons. Two-tailed t test; double asterisk, $p < 0.01$.

numbers of parvalbumin-expressing neurons were counted in the visual cortices of 10-week-old control and mutant mice. No obvious differences in morphology between these neurons in the *fBZ/fBZ* control and those in the *fBZ/fBZ;CaMKcre/+* conditional mutant were seen (data not shown). Counts of parvalbumin-positive interneurons are similar in control and mutant cortices (Figure 9G). Thus, these neurons do not appear to be affected by the loss of the TrkB receptor in neighboring cortical pyramidal neurons.

### Expression of ER81 Is Reduced Indirectly by Loss of the TrkB Receptor in the Neocortex

The dramatic alteration in dendritic arbors induced by *trkB* gene targeting in the neocortex could affect cortical circuits. Changes in circuit properties could alter gene expression and other properties of neurons, even those that continue to express TrkB. Changes in expression of an ETS transcription factor, ER81 (Lin et al., 1998), document an example of such indirect effects. In control animals, many layer V neurons express ER81 (Figure 10G). In a P44 *fBZ/+;CaMKcre/+* mouse, ~50% of ER81-positive layer V neurons express tau-β-galactosidase (Figures 10A–10C and 10J), indicating that ~50% of ER81-positive neurons lose TrkB in a P44 *CaMKcre*-directed *trkB* conditional mutant. Numbers of ER81-expressing neurons in both the visual cortex and somatosensory cortex are greatly reduced in the *fBZ/fBZ;CaMKcre/+* conditional mutant at the ages of 6 weeks and 10 weeks (Figures 10G–10I). Since detectable loss of *trkB* mutant neurons in the neocortex of the *trkB* conditional knockout is not seen in 6-week-old mice (Figures 5 and 8), the reduction in the number of ER81-positive neurons at 6 weeks appears to reflect loss or reduction of ER81 expression in a subset of layer V neurons.

To determine whether ER81 expression is affected specifically in neurons lacking TrkB, brain sections were costained with antibodies to both ER81 and β-galactosidase. If ER81 expression is lost specifically in *trkB* mutant neurons, the percentage of ER81-positive neurons that also express tau-β-galactosidase is expected to be greatly reduced in the *CaMKcre*-directed *trkB* conditional mutant. However, in both control (Figures 10A–10C) and mutant (Figures 10D–10F) mice at P44, many ER81-positive neurons also express tau-β-galactosidase. The percentage of ER81-positive neurons that also express β-galactosidase in *trkB* conditional mutant mice remains the same as that found in control mice (Figure 10J). These results indicate that expression of ER81 is affected equally in both *trkB* mutant neurons and TrkB-expressing neurons in the *trkB* conditional mutant. Thus, expression of ER81 is reduced nonspecifically or globally by the absence of TrkB in a subset of neurons in the *trkB* conditional mutant. Reduced expression does not appear to precede changes in dendritic morphology but does clearly occur before the period of major neuronal loss.

## Discussion

TrkB activation has been shown to be essential for the survival of many subpopulations of sensory neurons, including vestibular and nodose-petrosal neurons (reviewed by Reichardt and Fariñas, 1997), but no gross abnormalities have been reported in the brains of neonatal $trkB^{-/-}$ mice. The $trkB^{-/-}$ mice are not healthy, do not develop normally after birth, and do not survive longer than 2 to 3 weeks, making it very difficult to study the role of TrkB signaling in postnatal brain development and impossible to study its role in maintenance of the

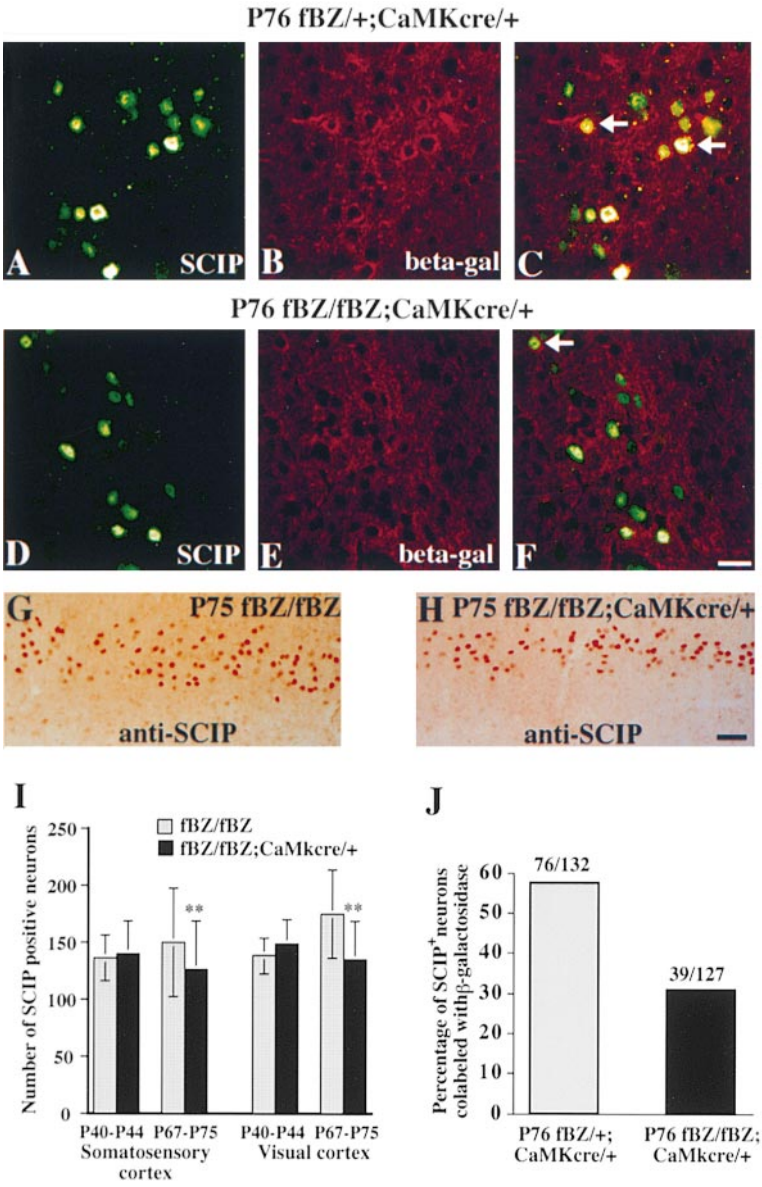


Figure 8. TrkB Activation Is Essential for Survival of Neurons Expressing SCIP at P76

(A–F) Colocalization of tau-β-galactosidase and SCIP. Many colabeled neurons were observed in *fBZ/+;CaMKcre/+* control animals (A–C), but only a very small percentage of SCIP-positive neurons also expressed tau-β-galactosidase in the *fBZ/fBZ;CaMKcre/+* conditional mutants (D–F). All confocal images were taken from the visual cortex region of P76 frozen coronal brain sections. Arrows in (C) and (F) indicate some of the colabeled neurons. Scale bar, 20 μm.

(G and H) Immunohistochemistry of SCIP in the somatosensory cortex region. Note that in the conditional mutant at P75, SCIP-expressing neurons are distributed in a narrower band. Scale bar, 50 μm.

(I) Counts of SCIP-expressing neurons in the visual cortex and somatosensory cortex. Neuronal counts were obtained from six to eight 730 μm wide stripes of sagittal sections from three pairs of *fBZ/fBZ* and *fBZ/fBZ;CaMKcre/+* brains at the age of P40–P44 and four pairs of *fBZ/fBZ* and *fBZ/fBZ;CaMKcre/+* brains at the age of P67–P75. Two-tailed paired t test; double asterisk, $p < 0.01$. Note the significant reduction in SCIP-expressing neuron development after P40.

(J) Percentage of SCIP-positive neurons that express tau-β-galactosidase. Counts were obtained from multiple brain sections of *fBZ/+;CaMKcre/+* and *fBZ/fBZ;CaMKcre/+* mice.

adult brain. To circumvent these problems, we have used the Cre/loxP recombination system to generate a chimeric *trkB* knockout in which loss of TrkB in the cortex is mostly restricted to pyramidal neurons. After loss of the TrkB receptor, which appears to be largely complete at 4 weeks, adult pyramidal neurons develop an altered morphology with reduced dendritic branches, thinner dendrites, and rounded cell soma at 6 weeks. At this age, there is obvious compression of layers II/III and V of the cortex. Over the following 4 weeks, many of these neurons eventually die or lose marker expression. Therefore, at 10 weeks, the effects of dendritic tree reduction and neuronal loss are both likely to contribute to neocortical compression.

### The Reporter Gene *tau-lacZ* in the Floxed *trkB* Locus Facilitates Analysis of the Function of TrkB in a Chimeric Neocortex

The gene-targeting technology based on the Cre/loxP recombination system makes it possible to create cell-type specific gene knockouts in essential genes. Cell-type specific mutants are extremely powerful tools for characterizing the functions of genes in particular cells in an otherwise healthy environment. In principle, two methods can be used to identify mutant cells in a chimeric tissue, either immunostaining for the gene product or introduction of a marker into the floxed allele. Particularly for cell surface proteins, such as TrkB, however, the presence of antigen in the surrounding cells makes it difficult to identify mutant cells with an antibody. Therefore, we introduced a reporter gene, *tau-lacZ*, into the floxed *trkB* allele in such a way that the *tau-lacZ* gene is expressed when Cre-mediated recombination deletes the *trkB* coding sequence. The marker tau-β-galactosidase allows us to identify easily neurons lacking TrkB. The presence of this marker has made it possible to characterize direct and indirect effects of the loss of the TrkB receptor on the morphology and survival of neurons. Adaptation of the design of the floxed allele described in this paper may be useful for the construc-

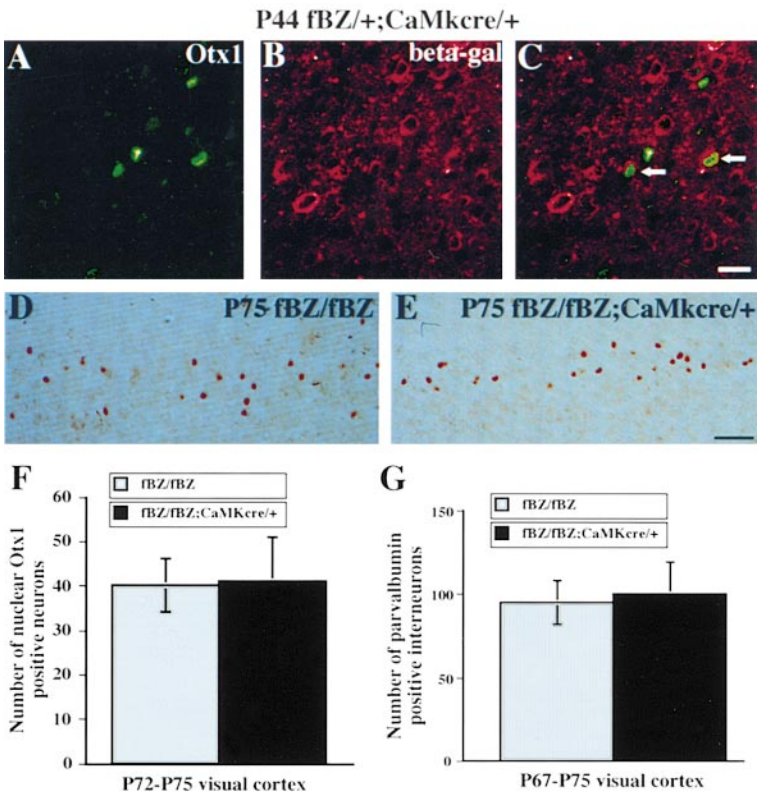


Figure 9. Survival of Neurons Expressing Nuclear Localized Otx1 or Parvalbumin Is Not Affected by *trkB* Targeting at P67–P75

(A–C) Colocalization of tau-β-galactosidase and Otx1. Colabeled neurons are indicated by arrows. Immunostaining was performed on frozen coronal brain sections. Scale bar, 20 μm.

(D and E) Immunohistochemistry of Otx1 in the visual cortex. Scale bar, 100 μm.

(F) Counts of nuclear Otx1-positive neurons in the visual cortex. Neuronal counts were obtained from six 1 mm wide stripes of sagittal sections from three pairs of *fBZ/fBZ* and *fBZ/fBZ;CaMKcre/+* brains with ages as indicated in the figure. Note that elimination of TrkB does not reduce the number of neurons with nuclear Otx1, although 46% (11 of 24) of these neurons were targeted.

(G) Counts of parvalbumin-expressing neurons. Neuronal counts were obtained from six 370 μm wide stripes of sagittal sections from three pairs of *fBZ/fBZ* and *fBZ/fBZ;CaMKcre/+* brains at the age of P67–P75. Note that absence of TrkB in cells expressing *CaMKcre* does not reduce the number of these neurons.

tion of modified alleles of other genes when one intends to examine the phenotypes of mutant cells in the presence of normal neighbors.

### The Morphology of Cortical Neurons Is an Early Target of the TrkB Receptor

One striking phenotype of the *CaMKcre*-mediated *trkB* knockout is simplification of the dendritic tree of cortical pyramidal neurons lacking TrkB. Apical dendrites are reduced in diameter, and some secondary dendrites are retracted or degenerated in the mutant neurons. The dendritic thinning can be detected as early as 4 weeks in mutant animals, while significant neuronal loss does not occur at or before 6 weeks of age (Figure 5). The retraction of dendrites is probably a major cause of the compression of the neocortex observed in 6-week-old and older mutants. As dendritic retraction precedes neuronal loss, it appears to be an early and specific phenotype caused by loss of TrkB. Interestingly, the shapes of cell bodies of $trkB^{-/-}$ cortical pyramidal neurons are also altered. Taken together, these results suggest that loss of the TrkB receptor results in alterations in the cytoskeletal structures of pyramidal neurons, which in turn induce changes in their morphologies.

It has been shown that the ligands for TrkB, BDNF, and NT-4/5 can alter dendritic growth of developing or nearly mature ferret cortical pyramidal neurons in slice culture (McAllister et al., 1995, 1997; Horch et al., 1999). In most cases, both BDNF and NT-4/5 increase the number of primary basal dendrites and the number of branches from both primary apical and basal dendrites. Extrapolating from these published works, one would expect fewer primary and higher order dendrites in murine cortical pyramidal neurons lacking TrkB. Consistent with this prediction, Sholl analyses demonstrate that murine cortical pyramidal neurons lacking TrkB have reduced dendritic complexity. Contrary to expectations, however, their primary dendritic number appears to be very similar to that of control neurons. In the chimeric murine cortex, visualization of β-galactosidase immunohistochemical staining indicates that there is dendritic retraction in mutant pyramidal neurons in all cortical layers. This result also appears somewhat different from observations of ferret slice cultures, where BDNF was shown actually to inhibit dendritic growth of developing neurons in layer VI, but not in other cortical layers (McAllister et al., 1997). There are so many differences between the two experimental systems, it is not surprising that there are some apparent discrepancies. For example, the studies of Katz and collaborators (McAllister et al., 1995, 1997; Horch et al., 1999) characterized changes in developing ferret cortex in vitro. Their work focused on the roles of neurotrophins in dendritic growth in immature neurons over the comparatively short span of 2 days. In contrast, our studies have characterized long-term changes in mature murine cortex in vivo. The *trkB* gene is not deleted in pyramidal cells in the *CaMKcre*-mediated *trkB* mutant until the third and fourth postnatal weeks, i.e., after substantial maturation of these neurons. The dendritic phenotype observed in our studies develops over a week or more. The longer time span of our studies may explain why additional effects on the morphology of the cell soma were observed in the present study that were not observed in the developing

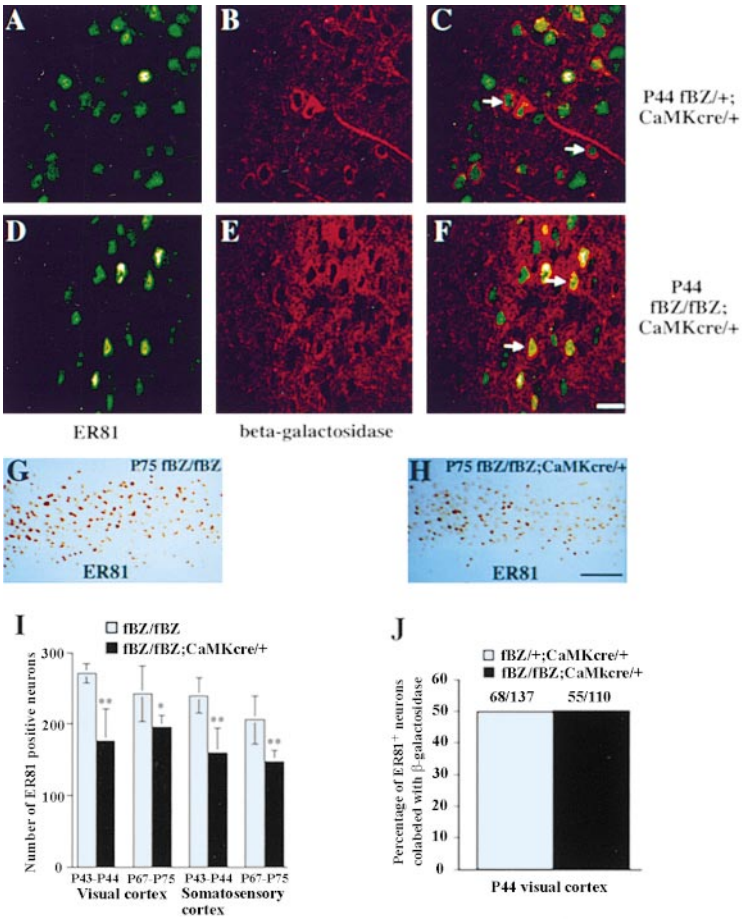


Figure 10. Expression of ER81 Is Reduced in the Neocortex of *trkB* Conditional Mutants

(A–F) Colocalization of tau-β-galactosidase and ER81. Many colabeled neurons were observed in both *fBZ/+;CaMKcre/+* control (A–C) and *fBZ/fBZ;CaMKcre/+* conditional mutant (D–F) mice. The confocal images were taken from the visual cortex region of P44 frozen coronal brain sections. Arrows in (C) and (F) indicate some of the colabeled neurons. Scale bar, 20 μm.

(G and H) Immunohistochemistry of ER81 in the visual cortex region. Scale bar, 100 μm.

(I) Counts of ER81-expressing neurons in the visual cortex and somatosensory cortex. Neuronal counts were obtained from six 550 μm wide stripes of sagittal sections from three pairs of *fBZ/fBZ* and *fBZ/fBZ;CaMKcre/+* brains with ages as indicated in the figure. Two-tailed t test; asterisk, $p < 0.05$; double asterisk, $p < 0.01$.

(J) Percentage of ER81 neurons that express tau-β-galactosidase. Counts were obtained from multiple brain sections of *fBZ/+; CaMKcre/+* and *fBZ/fBZ;CaMKcre/+* mice at P44.

ferret cortex. In future work, it will be interesting to examine the consequences of deleting *trkB* at earlier times in the developing murine cortex to determine if genetic ablation in vivo generates a phenotype that more closely resembles that described in the developing ferret cortex subjected to treatment with scavengers of TrkB ligands in vitro. We believe, though, that the major message of both studies is very similar: neurotrophin-mediated signaling regulates dendritic growth and structure in the neocortex. Since both full-length and truncated TrkB isoforms are removed in mutant neurons of our *trkB* condition mutant, it is unclear how much each isoform contributes to the alteration in neuronal morphology. In future work, this issue can be addressed by introducing the $trkB^{tk}$ mutant allele (Klein et al., 1993), which only expresses the truncated TrkB into the *fBZ* conditional mutant.

Synaptic plasticity is critical for development and proper function of the CNS. Formation and elimination of synapses are involved in some forms of synaptic plasticity, including ocular dominance column formation (reviewed by McAllister et al., 1999). Dendritic structure, especially the morphology and locations of spines, is modified by activity (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999). Expression of BDNF is regulated in neurons by depolarization and electrical activity (reviewed by McAllister et al., 1999). Moreover, results from this study and previous works (McAllister et al., 1995, 1997; Horch et al., 1999) show that neurotrophins regulate dendritic structure of both developing and mature cortical neurons. This regulation must surely affect the formation and stability of synapses, if not directly then indirectly, by removing the dendrites on which they are made. These observations suggest that neurotrophins can function as mediators of activity-dependent synaptic plasticity through regulation of dendritic structure.

### Specific Populations of TrkB-Expressing Cortical Neurons Require TrkB Signaling for Survival or Maintenance of Marker Expression

As the *CaMKcre*-directed *trkB* conditional mutants are viable, it has been possible to determine whether TrkB is essential for the survival of mature neurons in the neocortex and hippocampus. In this mouse, only a few neurons have deleted the *fBZ* allele before 2 weeks of age, making it possible to study TrkB function in mature neurons. Interestingly, even though our evidence argues that, in this mutant, pyramidal neurons are lost in the cortex, but not in the hippocampus, neuronal loss is not observed for several weeks after loss of TrkB function. Deletion of the floxed *trkB* locus appears to plateau in 4-week-old animals (Figure 2), and changes in dendritic morphology are seen also at this age (Figure 5), but significant neuronal loss has not been detected even 2 weeks later. Only at 10 weeks did our analyses detect significant losses of neurons expressing the reporter for

*trkB* elimination, tau-β-galactosidase, or the transcription factor SCIP. In contrast, in the embryonic PNS, neurons undergo apoptosis within a day in absence of a neurotrophin (Fariñas et al., 1996; Huang et al., 1999). Studies in cell culture have indicated that sensory neurons become progressively more resistant to loss of trophic factor support as their age increases (e.g., Acheson et al., 1995). In the future, it will clearly be important to identify the factors that explain these differences. It is important to note that it is possible that loss of tau-β-galactosidase- or SCIP-expressing neurons is due to downregulation of these marker genes, although our data suggest strongly that loss of these neurons results from cell death.

Our experiments argue that some neurons depend upon TrkB function cell autonomously. There is much more extensive loss, for example, of SCIP-expressing neurons, in which the *fBZ* allele has been targeted, than in similar neurons that continue to express TrkB. Despite this, it is not clear whether loss of these neurons is due directly to loss of cytoplasmic signals transmitted by the TrkB signaling or is, instead, a secondary effect of changes in dendritic morphology. As shown in culture studies of retinal ganglion neurons and motor neurons (e.g., Meyer-Franke et al., 1995), some CNS neurons require activity in addition to multiple trophic factors for survival. The changes in dendritic morphology in pyramidal cells induced by loss of TrkB almost certainly reduce their innervation by other neurons, which in turn is expected to affect their electrical activity and access to other trophic factors. Interestingly, the dendritic structure of CA1 pyramidal neurons is not obviously affected by loss of TrkB, and, concomitantly, the survival of these neurons does not appear to be dependent on TrkB signaling, at least over the time span of 10 weeks (Figures 7E and 7F; B. Xu et al., submitted). In addition, our data show that a small population of layer V neurons that have the transcription factor Otx1 localized in their nuclei is also not obviously dependent upon TrkB signaling for survival. For technical reasons, we only examined the dendritic trees of layers II/III in the present study, which did not include this population. In future work, it will be interesting to determine whether TrkB loss perturbs the dendritic trees of these neurons.

### Similarity of Cortical Degeneration in the *CaMKcre*-Mediated *trkB* Mutant to Neurodegenerative Disease Models

Neurodegenerative diseases are characterized by death of selective populations of neurons and gliosis. Experimental animal models have been developed for several neurodegenerative diseases, including spinocerebellar ataxia type 1 (SCA1) (Burright et al., 1995), Huntington's disease (Reddy et al., 1998), and Alzheimer's disease (Games et al., 1995). There are some striking similarities between the cortical phenotype in the *CaMKcre*-mediated *trkB* conditional mutant and the pathologies present in many neurodegenerative diseases. In both cases, selective populations of neurons die progressively, and reactive astrocytosis (data not shown) is observed in the affected regions. In the SCA1 mouse disease model, degeneration of dendritic trees precedes the death of cerebellar Purkinje neurons (Burright et al., 1995). It has been shown that dendritic trees of Purkinje neurons are altered in *BDNF* mutant mice, although the mutant does not survive long enough to examine whether Purkinje neurons eventually die (Schwartz et al., 1997). Our results demonstrate that changes in the morphology of dendrites precede the loss of cortical pyramidal neurons in the absence of TrkB signaling. Because of these similarities, it will be interesting to assess the role of receptor tyrosine kinase–mediated signals in the progression of these diseases.

## Experimental Procedures

### Generation of *CaMKcre*-Directed *trkB* Mutants

*CaMKcre*-directed *trkB* conditional mutants and control mice were generated as described (B. Xu et al., submitted). In brief, the *fBZ* heterozygous mice were bred with *CaMKcre* transgenic mice to generate mice heterozygous for both the *fBZ* allele and the *CaMKcre* transgene (*fBZ/+;CaMKcre/+*). The double heterozygous mice were mated with *fBZ* heterozygotes (*fBZ/+;+/+*) to obtain *trkB* conditional knockouts (*fBZ/fBZ;CaMKcre/+*) and their control animals. Animals heterozygous for both *fBZ* and *CaMKcre* were also used to analyze the pattern of Cre-mediated *trkB* knockout in the brain by using X-gal staining and β-galactosidase immunohistochemistry. The $trkB^{lacZ}$ allele was generated by crossing a *cre* transgene under the control of the β-actin promoter into *fBZ/+* mice. Genotypes of mice were determined by PCR or Southern hybridization. Animals were maintained under standard conditions.

### Histological Methods

For frozen sections, animals were anesthetized and transcardially perfused with 20 ml of phosphate-buffered saline (PBS), 40 ml of 4% paraformaldehyde in PBS, and 20 ml of PBS. The brains were cryoprotected in 30% sucrose, embedded in O. C. T. medium, and stored at −80°C. The frozen brains were sectioned at 20 μm sagittally or coronally in a cryostat and processed for X-gal and immunofluorescence staining as described (Fariñas et al., 1996).

For other histological staining, animals were anesthetized and transcardially perfused with 20 ml of PBS and 40 ml of 4% paraformaldehyde in PBS. The brains were postfixed in 4% paraformaldehyde for 6–16 hr. Sagittal sections at 50 μm were obtained with a vibratome and collected in PBS. Nissl staining and immunohistochemistry were performed as described (Fariñas et al., 1996). The monoclonal antibody to Otx1 is described in Weimann et al. (1999). Monoclonal antibodies to calbindin (1:1000) and parvalbumin (1:1000), and polyclonal antibodies to glial fibrillary acidic protein (1:400), were from Sigma Chemicals. Antibodies to β-galactosidase were purchased from Promega Life Science (monoclonal, 1:250) and ICN Pharmaceuticals (rabbit polyclonal, 1:3000). Polyclonal antibodies to SCIP and polyclonal antibodies to ER81 were provided by Dr. Greg Lemke (Salk Institute, La Jolla, CA) and Drs. Silvia Arber and Thomas Jessell (Columbia University, New York, NY), respectively. Monoclonal antibodies to the α subunit of CaMKII (1:100) were purchased from Affinity Bioreagents.

### Neuronal Counts

Sagittal sections from the same ages of control and mutant mice (littermates in most cases) were processed for immunohistochemistry. Neurons were photographed from a stripe of sagittal section at the area of interest. Two stripes were imaged from each of three to four animals, and only one stripe was taken from each of two sections that were 0.6 mm away from each other laterally. All stripes for neuronal counts were location matched between control and mutant animals. Neurons were counted on prints blindly. Neuronal counts were then matched with genotypes by a different person. For β-galactosidase-immunostained sections, only positive neurons with clear nuclei were counted.

### Western Blots

The TrkB antibodies (RTB) for Western blot were raised to the TrkB extracellular domain (Huang et al., 1999). The monoclonal antibody

to α-tubulin was purchased from Sigma Chemicals. Western blots were quantified using a Fuji Multiimager.

### NMDA/Biocytin Injections

Anesthesia of mice was initiated with 2.5% isoflurane in 100% oxygen at 1 l/min, followed by an intraperitoneal injection of 7 μl/g of 2.5% avertin. Animals were placed in a stereotactic apparatus, and heart rate was monitored and kept at an anesthetic level of ~400 beats/min with 0.5%–1.0% isoflurane/1% oxygen. Atropine (30 μl, 10 mg/ml) and dexamethasone (50 μl, 4 mg/ml) were administered subcutaneously. An incision was made in the midline of the scalp, and the scalp was retracted with clips. The fascia was removed from the skull with a scalpel blade, and a small craniotomy was made unilaterally over the area 17/18 border. A slit was made in the dura mater with the tip of a 30 gauge needle.

Injections were made using a variation of the method described by Jiang et al. (1993). A glass pipette with a 30–50 μm diameter tip was filled with silicone oil for use in a Nanoject II injector (Drummond Scientific Company, Broomall, PA), after which 10 mM NMDA in PBS was drawn up into the tip. The pipette was lowered to a depth of 400 μm below the pial surface, and six injections of 23 nl were made at 30 s intervals. The pipette was removed, and a glass pipette (tip diameter, 5–10 μm) filled with 4%–5% biocytin in 50 mM Tris–Cl (pH 7.6) was lowered into the same position. The biocytin was iontophoretically injected at 5 μA for 12 min. Shorter injection times were sometimes used if the animal's breathing became labored; these yielded smaller injections that were qualitatively similar to the larger injections made by longer iontophoresis. The scalp was sutured and the animal recovered.

After a 24 hr survival period, the mice were overdosed with Nembutal and perfused transcardially with 0.1 M Na phosphate buffer (PBS), followed by 4% paraformaldehyde in PBS. The brain was removed from the skull and postfixed overnight. The brain was embedded in gelatin/albumin and cut at 75 μm on a vibratome. Sections were blocked in Tris-buffered saline, 3% horse serum, 3% bovine serum albumin, and 0.4% Triton X-100 at room temperature for 1 hr, then incubated in the same solution plus mouse anti-β-galactosidase monoclonal antibodies (1:250, Promega, Madison, WI) overnight at room temperature. After several washes, the sections were incubated with fluorescein-conjugated horse anti-mouse IgG antibodies (Vector, Burlingame, CA) and Texas Red–conjugated avidin (Vector) for 3–4 hr at room temperature. After extensive washes, the sections were mounted on slides and coverslipped with Vectashield antifade mounting medium (Vector).

Neurons that were filled by biocytin and well isolated from other filled neurons were examined for expression of β-galactosidase. Neurons that are positive for both biocytin and β-galactosidase were randomly selected and imaged by using a confocal microscope. For each neuron, two Z series of confocal images at a 2 μm step were taken to cover most of dendrites of the neuron (usually 25–35 optical sections). The dendritic tree of the neuron was reconstructed by projection of a Z series of images and analyzed according to the Sholl method (Sholl, 1953).

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