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Loss of TGF-β1 Leads to Increased Neuronal Cell Death and Microgliosis in Mouse Brain

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Summary

TGF-β1 is a key regulator of diverse biological processes in many tissues and cell types, but its exact function in the developing and adult mammalian CNS is still unknown. We report that lack of TGF-β1 expression in neonatal *Tgfb1*$^{-/-}$ mice results in a widespread increase in degenerating neurons accompanied by reduced expression of synaptophysin and laminin and a prominent microgliosis. Lack of TGF-β1 also strongly reduces survival of primary neurons cultured from *Tgfb1*$^{-/-}$ mice. TGF-β1 deficiency in adult *Tgfb1*$^{-/+}$ mice results in increased neuronal susceptibility to excitotoxic injury, whereas astroglial overexpression of TGF-β1 protects adult mice against neurodegeneration in acute, excitotoxic and chronic injury paradigms. This study reveals a nonredundant function for TGF-β1 in maintaining neuronal integrity and survival of CNS neurons and in regulating microglial activation. Because individual TGF-β1 expression levels in the brain vary considerably between humans, this finding could have important implications for susceptibility to neurodegeneration.

Introduction

Transforming growth factor-β (TGF-β) superfamily members, including TGF-βs, bone morphogenic proteins (BMPs), activins, glial derived neurotrophic factor (GDNF), and nodal, fulfill key functions during development and in maintaining tissue homeostasis (reviewed in Dennler et al., 2002; Massagué et al., 2000). In the CNS, BMPs control neuronal, astroglial, and oligodendroglial cell induction, neuronal cell-type specification, and patterning (reviewed in Ebendal et al., 1998; Mehler et al., 1997). In contrast, little is known about the role of TGF-βs in the CNS.

The TGF-β subfamily includes three isoforms in mammals, TGF-β1, 2, and 3. They act in a highly contextual manner, and depending on cell type and environment, TGF-βs may promote cell survival or induce apoptosis, stimulate cell proliferation or induce differentiation, and initiate or resolve inflammation (Dennler et al., 2002; Massagué et al., 2000; Roberts and Sporn, 1996). Consequently, disruption of TGF-βs results in severe isoform-specific phenotypes and usually embryonic lethality in mice (Letterio, 2000; Weinstein et al., 2000). The diverse effects of all three TGF-β isoforms are transduced through the same type I and type II heterodimeric transmembrane serine/threonine kinase receptors. Ligand binding results in receptor phosphorylation and recruitment of Smad proteins, which translocate to the nucleus where they associate with other transcription factors to regulate gene transcription (Dennler et al., 2002; Massagué et al., 2000). How exactly specificity of signaling is achieved is still unknown, but temporal and spatial regulation of TGF-β gene expression, differential binding affinity to its receptor, and an array of extracellular binding molecules for TGF-βs and intracellular signaling molecules participate in the integration of TGF-β signaling and the generation of an appropriate biological response (Dennler et al., 2002; Massagué et al., 2000).

TGF-βs are probably expressed in most cell types in vivo, and at least in culture almost all cells can synthesize the TGF-β1 isoform (Roberts and Sporn, 1996). The TGF-β1 promoter is activated by wounding, ischemia, and cellular stress in general (Kim et al., 1990; Roberts and Sporn, 1996). Accordingly, TGF-β1, which is normally present at low levels in healthy adult CNS cells, is rapidly upregulated after injury and induces expression of many injury response genes (Finch et al., 1993). Cerebral TGF-β1 expression is also increased with age (Finch et al., 1993). The main sources of TGF-β1 in the injured brain are astrocytes and microglia (Finch et al., 1993), but neurons can produce it as well (Flanders et al., 1998). TGF-β2 and TGF-β3 are regulated mainly by hormonal and developmental signals, and it has been speculated that they have a role in CNS development (Flanders et al., 1998).

Numerous studies used cultured neurons to show a protective effect of TGF-β1 against various toxins and injurious agents (reviewed in Flanders et al., 1998; Unsicker and Krieglstein, 2002). Similar results were obtained when TGF-βs were delivered intracerebrally or using viral vectors into adult rodent brains after ischemic injury (Unsicker and Krieglstein, 2002). In contrast, in vivo neutralization of TGF-βs with antibodies during early CNS development prevented ontogenetic neuronal death in the chick embryo cilliary ganglion and retina (Dünker et al., 2001; Krieglstein et al., 2000), indicating that TGF-βs can also trigger neuronal cell death under certain conditions. Other support for an important role for TGF-βs and the TGF-β signaling pathway in neuronal development and function comes from recent experiments in *Drosophila* and *Aplysia* (Chin et al., 2002; Sweeney and Davis, 2002; Zhang et al., 1997). In *Drosophila*, TGF-β receptors and dSmad2 are required for neuronal remodeling (Zheng et al., 2003). Mutations in *spinster*, resulting in enhanced TGF-β signaling, caused synaptic overgrowth, which could be reverted by down-regulation of TGF-β signaling (Sweeney and Davis, 2002). In *Aplysia* sensory neurons, TGF-β1 induces

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phosphorylation and redistribution of the presynaptic protein synapsin and modulates synaptic function (Chin et al., 2002; Zhang et al., 1997). However, none of the above studies could address whether TGF-β1 is required for neuronal development and survival in the mammalian CNS and how it exerts its actions.

Here we used mice deficient in TGF-β1 and primary neuronal cultures from these mice to study the role of TGF-β1 in mammalian CNS development and injury. C57BL/6 mice lacking TGF-β1 have defects in vasculogenesis and angiogenesis leading to early embryonic lethality at E9.5 without overt defects in brain development at this early stage (Dickson et al., 1995; Kulkarni et al., 1993). However, the NIH genetic background rescues *Tgfb1*$^{-/-}$ mice from embryonic lethality (Bonyadi et al., 1997). These mice have no overt developmental abnormalities but succumb to an autoimmune wasting syndrome at 3–4 weeks of age (Bonyadi et al., 1997). We found that unmanipulated neonatal *Tgfb1*$^{-/-}$ mice show increased numbers of apoptotic neurons, a reduction in neocortical presynaptic integrity, reduced expression of the extracellular matrix protein laminin, and widespread microgliosis. Cultured primary neurons lacking TGF-β1 have reduced survival compared with wild-type controls. Heterozygous knockout (*Tgfb1*$^{-/+}$) mice, which have normal life spans, show increased susceptibility to excitotoxic injury and neurodegeneration, whereas transgenic overproduction of TGF-β1 prevented degeneration after excitotoxic injury. We conclude that TGF-β1 has a nonredundant function in maintaining neuronal integrity and survival of CNS neurons and in regulating microglial activation.

Results

Cerebral Overexpression of TGF-β1 Protects Against Acute and Chronic Neuronal Injury

To determine whether increased production of endogenous bioactive TGF-β1 from astrocytes could protect against acute neuronal injury, we used 2- to 4-month-old mice expressing TGF-β1 under control of the glial fibrillary acidic protein (GFAP) promoter 2-fold (low, line T64) or 4-fold (medium, line T115) above endogenous TGF-β1 mRNA levels (Wyss-Coray et al., 1995, 2000). Heterozygous TGF-β1 transgenic mice and nontransgenic littermate controls were injected intraperitoneally with kainic acid (KA) in an experimental model of excitotoxic neuronal injury (Schauwecker and Steward, 1997). Excitotoxicity has been postulated to be a major mechanism of neuronal degeneration in stroke, Alzheimer disease (AD), and other neurodegenerative diseases (Rothman and Olney, 1995). The mice used here overexpressing TGF-β1 at low levels did not develop signs of hydrocephalus as reported for high expressor mice (Wyss-Coray et al., 1995), and KA treatment did not cause overt changes in brain architecture (data not shown). However, KA treatment resulted in a 24% loss of MAP-2 immunoreactivity (Figure 1A) and a 60% loss in the number of pyramidal cells expressing calbindin (Figure 1B) in the neocortex (this loss in calbindin immunoreactivity is not accompanied by cell death [data not shown]). TGF-β1 overexpression in astrocytes at low or medium levels significantly reduced the dendritic damage (Figure 1A). Overexpression at medium levels also prevented the loss in calbindin immunoreactivity in pyramidal cells in the neocortex (Figure 1B). Although not significantly different from nontransgenic mice, there is a trend toward higher numbers of calbindin-positive neurons in the noninjured TGF-β1 low expresser mice (Figure 1B).

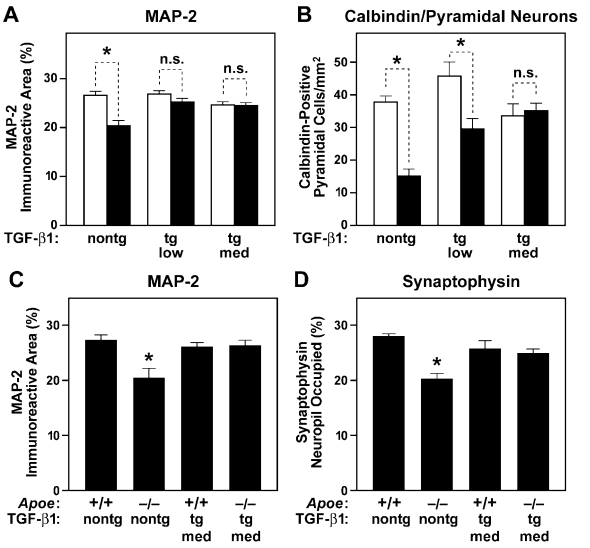

Figure 1. TGF-β1 Overexpression in Astrocytes Protects Against Injury-Induced Neurodegeneration

(A and B) TGF-β1 overexpression in astrocytes of TGF-β1 tg mice significantly reduced the loss of MAP-2 immunoreactivity (A) and calbindin-positive pyramidal cells (B) induced by KA in the neocortex. Sagittal brain sections of 2- to 4-month-old TGF-β1 heterozygous transgenic low (tg low) and medium (tg med) expressors and nontransgenic littermate control (nontg) mice injected with 25 mg/kg KA (black bars) or PBS (white bars) were stained for MAP-2, and the percent immunoreactive area of the neuropil was determined by confocal microscopy and computer-aided image analysis or immunolabeled for calbindin and the numbers of neurons were counted. Values are mean ± SEM, *$p < 0.05$; n.s., not significant, Tukey-Kramer test, 4–5 mice per genotype and treatment.

(C and D) TGF-β1 overexpression in astrocytes of *Apoe*$^{-/-}$ mice significantly reduced the loss of MAP-2 (C) and synaptophysin (D) immunostaining in the neocortex as a result of the lack of apoE. Sagittal brain sections of 9- to 10-month-old nontransgenic (nontg), *Apoe*$^{-/-}$, heterozygous TGF-β1 transgenic medium expressor (tg med), and TGF-β1 tg med, *Apoe*$^{-/-}$ mice were immunolabeled for MAP-2 and synaptophysin, and relative expression of these proteins was quantified as described above. Values in TGF-β1 nontg, *Apoe*$^{-/-}$ mice (indicated by *) are significantly different from all other groups in (C) and (D). Values are mean ± SEM, *$p < 0.01$, Tukey-Kramer test, 4–5 mice per genotype.

To determine the effect of TGF-β1 on chronic neuronal injury, we crossed *Apoe* knockout mice (*Apoe*$^{-/-}$), which show an age-dependent loss of synaptophysin-positive presynaptic terminals and neuronal cytoskeletal abnormalities (Masliah et al., 1995), with TGF-β1 mice (line T115). No overt morphological changes were observed in any of the resulting offspring (data not shown). As described previously (Masliah et al., 1995), 9- to 10-month-old mice lacking *Apoe* expression showed a roughly 30% loss in MAP-2 immunostaining and synaptophysin-positive terminals compared with wild-type mice (Figures 1C and 1D). TGF-β1 overexpression at

Table 1. Genotype and Weight Distribution in TGF-β1-Deficient and Wild-Type Mice

Tgfb1 Genotype		+/+	−/+	−/−
Genotype frequency [% (n)][a]	P1	25.0 (15)	51.7 (31)	23.3 (14)
	P21	29.5 (39)	46.2 (61)	17.4 (23)
	adult	39.1 (182)	60.9 (283)	N/A[b]
Body weight [g (n)]	P1	1.4 ± 0.1 (3)	1.5 ± 0.03 (10)	1.4 ± 0.1 (7)[c]
	P21	12.6 ± 0.9 (14)	13.3 ± 0.6 (21)	8.5 ± 0.6 (13)[d]
	adult	30.9 ± 1.3 (14)	29.6 ± 1.7 (12)	N/A[b]
Hemibrain weight [mg (n)]	P1	58.5 ± 4.8 (3)	50.3 ± 4.6 (13)	44.4 ± 2.7 (4)[c]
	P21	224.4 ± 7.4 (9)	229.9 ± 4.6 (12)	197.8 ± 16.4 (5)[c]
	adult	239.1 ± 4.2 (3)	223.6 ± 11.8 (4)	N/A[b]

[a] Percentage of mice surviving up to indicated age. Adult, 2- to 4-month-old.
[b] No survivors at this age.
[c] Not significantly different from $Tgfb1^{+/+}$.
[d] $p < 0.01$, Tukey-Kramer test.

medium levels (line T115) largely prevented this loss (Figures 1C and 1D). TGF-β1, $Apoe^{+/+}$ mice did not differ from their nontransgenic littermates, suggesting that higher levels of TGF-β1 do not increase the number of synaptophysin-positive synaptic connections and dendrites above normal levels and do not prevent normal age-related decreases in these proteins. Taken together, TGF-β1 overexpression largely prevents the loss of synaptic and/or dendritic markers as well as the reduction in neuronal calbindin expression after chronic and/or acute injury.

TGF-β1-Deficient Mice Have Increased Susceptibility to Kainic Acid-Induced Neuronal Injury

To test whether TGF-β1 is not only sufficient but also necessary to protect neurons against acute neuronal injury, we injected 5- to 6-month-old mice lacking one copy of the *Tgfb1* gene ($Tgfb1^{-/+}$) and wild-type ($Tgfb1^{+/+}$) mice with KA. On the NIH/Ola background, $Tgfb1^{-/+}$ mice show no gross abnormalities (Bonyadi et al., 1997) and have similar brain and body weights compared with $Tgfb1^{+/+}$ mice (Table 1). At 3 weeks (Figure 2A) or 10 months of age (data not shown), unmanipulated $Tgfb1^{-/+}$ mice express approximately half the TGF-β1 mRNA compared with $Tgfb1^{+/+}$ littermates. $Tgfb1^{-/-}$ mice express no TGF-β1 mRNA (Figure 2A). Levels of TGF-β2 mRNA in the brain were also not significantly different between 10-month-old $Tgfb1^{-/+}$ and $Tgfb1^{+/+}$ littermate controls (6.4 ± 1.6 versus 7.2 ± 1.5; $p = 0.7$; $n = 10$ mice per genotype). Others have reported that TGF-β2 and TGF-β3 mRNAs are not altered in heart or lung of $Tgfb1^{-/-}$ mice (Kulkarni et al., 1993).

Twenty-four hours after KA injection, 5- to 6-month-old $Tgfb1^{-/+}$ mice expressing reduced levels of endogenous TGF-β1 showed a 17-fold increase in the number of caspase-positive cells compared with $Tgfb1^{+/+}$ littermate controls (Figure 2B). All mice seized similarly and reached a minimum of stage 3 on a 0 to 5 seizure scale (see Experimental Procedures; data not shown), suggesting that this increase was not caused by differences in drug kinetics or availability in the two genotypes. The vast majority of caspase-positive cells were neurons (Figure 2C). They were observed in the neocortex, hippocampus, caudate, putamen, amygdala, and thalamus. Only few caspase-positive cells were found in $Tgfb1^{+/+}$ mice (Figure 2B), indicating that the NIH mouse strain is relatively resistant to excitotoxic neuronal damage.

Consistent with the activated caspase labeling, 5- to 6-month-old KA-injected $Tgfb1^{-/+}$ mice showed a 6-fold increase in the number of cells displaying terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) compared with $Tgfb1^{+/+}$ littermate controls. Due to great interindividual variability in the $Tgfb1^{-/+}$ mice, this difference was not statistically significant (26.33 ± 8.59 versus 157.60 ± 77.49; $p < 0.2$, Mann-Whitney U test, 5–6 mice per genotype). TUNEL colocalized largely with the neuronal marker NeuN (Figure 2D), indicating that neurons undergo apoptotic changes in KA-lesioned, TGF-β1-deficient mice. Although more cells were caspase positive than TUNEL positive, areas with staining for the two markers overlapped, and relative numbers of TUNEL- and caspase-positive cells correlated positively across both genotypes ($r = 0.984$, $p < 0.001$, 5–6 mice per genotype). This finding is consistent with the two markers representing two aspects of the same apoptotic events and TUNEL identifying more severely damaged neurons than the caspase marker.

Unmanipulated $Tgfb1^{-/-}$ Mice Display Neuronal Degeneration

To test whether lack of TGF-β1 expression further increases susceptibility to neuronal cell death and degeneration, 1-day-old (P1) or 21-day-old (P21) $Tgfb1^{-/-}$ mice were analyzed and compared with $Tgfb1^{-/+}$ and $Tgfb1^{+/+}$ littermate controls. $Tgfb1^{-/+}$ breedings resulted in the expected frequency of $Tgfb1^{-/-}$ mice (Table 1) on the NIH/Ola background (Bonyadi et al., 1997). Body and brain weights were normal at P1, but $Tgfb1^{-/-}$ mice weighed around 30% less than $Tgfb1^{-/+}$ and $Tgfb1^{+/+}$ littermates at 3 weeks of age (Table 1), as a result of the peripheral wasting syndrome in these mice (Geiser et al., 1993; Kulkarni et al., 1993; Shull et al., 1992). Brains of $Tgfb1^{-/+}$ mice at all ages had a tendency to weigh less than those of control littermates, although this did not reach statistical significance (Table 1). Neocortices of P21 $Tgfb1^{-/-}$ mice appeared compacted and thinner than $Tgfb1^{+/+}$ cortices on histological sections (Figure 3), but staining with H&E, cresyl violet (Figures 3A and 3B), or NeuN (data not shown) showed no gross

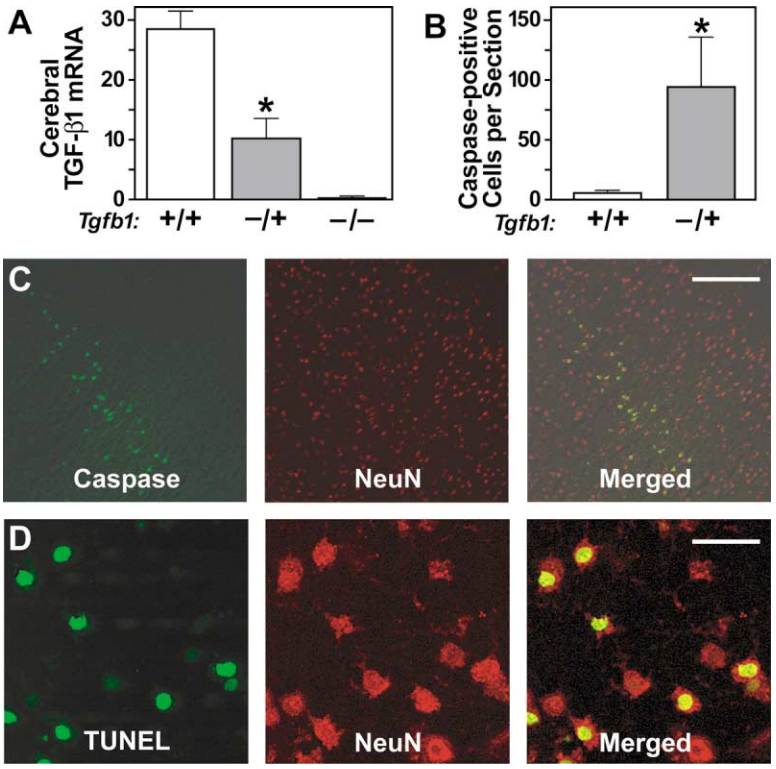

Figure 2. *Tgfb1*$^{-/+}$ Mice Display More Caspase- and TUNEL-Positive Neurons than *Tgfb1*$^{+/+}$ Mice following Excitotoxic Injury

(A) Cerebral TGF-β1 mRNA levels in unmanipulated P21 *Tgfb1*$^{+/+}$, *Tgfb1*$^{-/+}$, and *Tgfb1*$^{-/-}$ mice as determined by RNase protection assay. Specific signals were quantified and normalized to β-actin mRNA as a control. Values are mean ± SEM, *p < 0.01, Tukey-Kramer test, 3–4 mice per genotype.

(B) Coronal brain sections of 5- to 6-month-old *Tgfb1*$^{+/+}$ and *Tgfb1*$^{-/+}$ mice injected with 15 mg/kg KA were labeled with a FITC-tagged panspecific inhibitor of activated caspases, and the number of positive cells per section was counted. Values are mean ± SEM, *p < 0.009, Mann-Whitney U test, 5–6 mice per genotype.

(C and D) Double labeling and confocal microscopy of coronal brain sections from KA-injected *Tgfb1*$^{-/+}$ mice identified most cells positive for activated caspases (C) or TUNEL (D) as neurons using the neuronal nuclear protein NeuN. Representative cortical areas are shown. Scale bar, 200 μm (C) and 30 μm (D).

abnormalities in brain architecture. Consistent with the reported effects of TGF-β1 on blood vessel formation (Dickson et al., 1995), *Tgfb1*$^{-/-}$ mice showed abnormally distended cortical blood vessels at P1, but they were not observed at P21 or in *Tgfb1*$^{-/+}$ and *Tgfb1*$^{+/+}$ mice (data not shown).

To determine whether loss of TGF-β1 results in increased neuronal cell death or degeneration, brain sec-

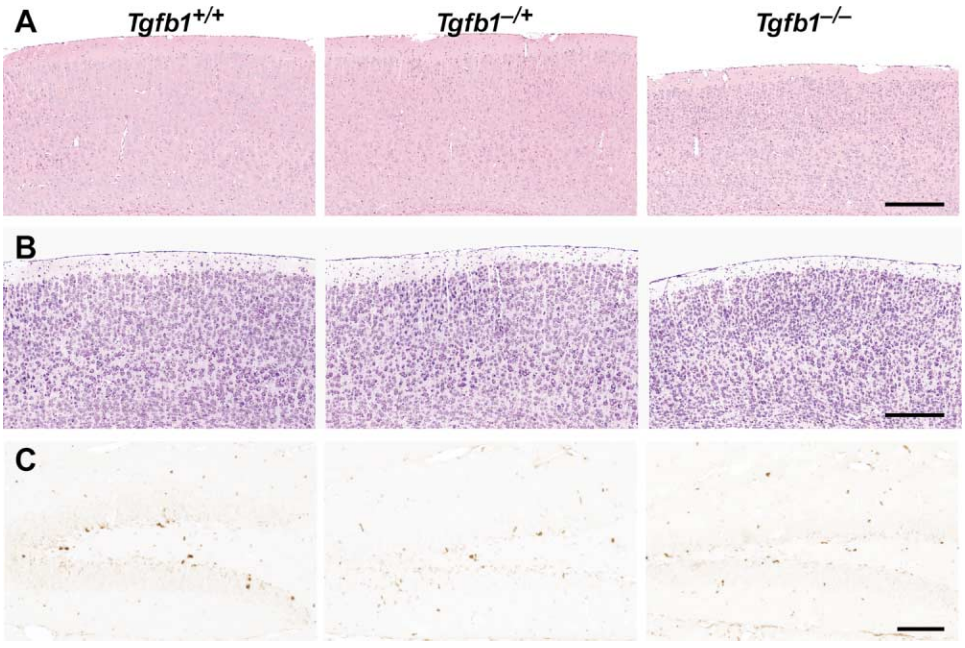

Figure 3. *Tgfb1*$^{-/-}$ Mice Show No Gross Structural Abnormalities in the Brain

(A and B) Sagittal brain sections of unmanipulated P21 *Tgfb1*$^{+/+}$, *Tgfb1*$^{-/+}$, and *Tgfb1*$^{-/-}$ mice were stained with eosin & hematoxylin (A) or cresyl violet (B). While no apparent damage could be observed, the neocortex of *Tgfb1*$^{-/-}$ mice seemed thinner and condensed (A and B).

(C) Immunolabeling for PCNA revealed no obvious differences in cell proliferation, as illustrated here in the dentate gyrus. Scale bar, 250 μm (A and B) and 50 μm (C).

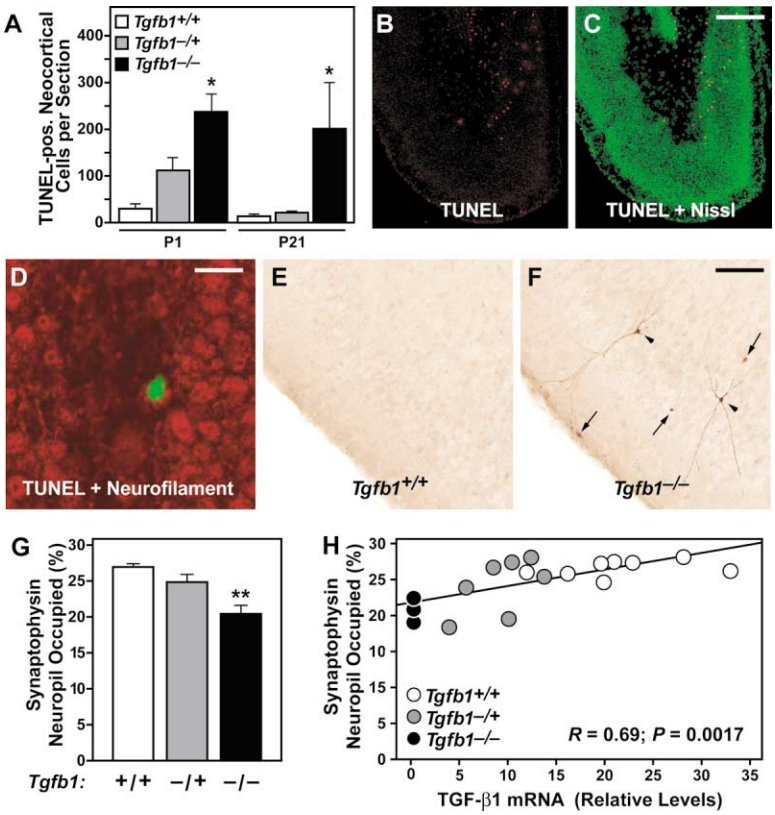

Figure 4. Absence of TGF-β1 Results in Neuronal Damage and Reduced Synaptic Density

(A) Sagittal brain sections of P1 and P21 unmanipulated *Tgfb1*$^{+/+}$, *Tgfb1*$^{-/+}$, and *Tgfb1*$^{-/-}$ mice labeled for TUNEL showed an increase in the number of positive cells per area in the neocortex of *Tgfb1*$^{-/-}$ mice. Values are means ± SEM, *$p < 0.05$, Tukey-Kramer test, 3–5 mice per genotype.

(B–D) Double labeling and confocal microscopy of coronal brain sections from P1 *Tgfb1*$^{-/-}$ mice identified most TUNEL-positive cells as neurons using green fluorescent Nissl labeling with TUNEL in red (B and C) or using neurofilament immunolabeling in red with TUNEL in green (D).

(E and F) Coronal brain sections of unmanipulated P21 *Tgfb1*$^{+/+}$ (E) and *Tgfb1*$^{-/-}$ (F) mice were immunolabeled for activated caspase 3. Positive neurons in the neocortex with long processes (arrowheads) were only observed in *Tgfb1*$^{-/-}$ mice. Arrows point to condensed nuclei or nonneuronal caspase 3-positive cells.

(G and H) Sagittal brain sections of unmanipulated P21 *Tgfb1*$^{+/+}$, *Tgfb1*$^{-/+}$, and *Tgfb1*$^{-/-}$ mice were immunolabeled for synaptophysin, the percent immunoreactive area of the neuropil was determined by confocal microscopy and computer-aided image analysis (G) and correlated with relative TGF-β1 mRNA levels measured in opposite hemibrains (H). Each symbol represents one mouse. Values are means ± SEM, **$p < 0.05$, Tukey-Kramer test, 3–8 mice per genotype.

Scale bar, 200 μm (C), 20 μm (D), and 120 μm (F).

tions from *Tgfb1*$^{-/-}$, *Tgfb1*$^{-/+}$, and *Tgfb1*$^{+/+}$ littermates were analyzed by TUNEL and synaptophysin staining. P1 *Tgfb1*$^{-/-}$ mice showed prominent TUNEL staining in different parts of the brain, including the neocortex (Figures 4B and 4C), caudate putamen, and cerebellum. In the neocortex, *Tgfb1*$^{-/-}$ mice had significantly more TUNEL-positive cells than *Tgfb1*$^{+/+}$ littermates at P1 and P21 (Figures 4A–4C). Some TUNEL-positive cells were also observed in *Tgfb1*$^{+/+}$ mice, which is consistent with neuronal cell death due to active neurogenesis during the first postnatal week in mice (Johnson and Deckwerth, 1993). *Tgfb1*$^{-/+}$ mice had a trend toward increased numbers of TUNEL-positive neurons at P1 but not P21 (Figure 4A). Most of the TUNEL-positive cells were identified as neurons by colocalization with fluorescent Nissl stain (Figures 4B and 4C) or by colocalization of TUNEL and neurofilament immunostaining and analysis by laser scanning confocal microscopy (Figure 4D). Furthermore, immunostaining with an antibody against activated caspase 3 labeled neurons with long processes in P21 *Tgfb1*$^{-/-}$ but not *Tgfb1*$^{+/+}$ littermates (Figures 4E and 4F). To determine whether the increase in TUNEL-positive neurons was the result of increased proliferation or delayed maturation of neuronal precursors, we stained brain sections with doublecortin (data not shown), a marker of immature neurons (Gleeson et al., 1998), or PCNA, a nuclear proliferation marker (Figure 3C). No obvious differences in staining intensities or distribution of these markers were observed between *Tgfb1*$^{-/-}$ mice and *Tgfb1*$^{+/+}$ littermate controls, indicating no gross abnormalities in neuronal maturation and cellular proliferation. Besides the increase in TUNEL-positive neurons, unmanipulated 3-week-old *Tgfb1*$^{-/-}$ mice showed also significantly less synaptophysin-positive synapses in the neocortex (Figure 4G) and hippocampus (data not shown) compared to *Tgfb1*$^{-/+}$ and *Tgfb1*$^{+/+}$ littermate controls, and relative levels of cerebral TGF-β1 mRNA correlated positively with the area of the cortex occupied by synaptophysin-positive presynaptic terminals (Figure 4H). This reduction in synaptophysin immunoreactivity was not restricted to the NIH genetic background. *Tgfb1*$^{-/+}$ mice (12- to 14-months-old) on the C57BL/6 background had significantly less synaptophysin immunostaining in the neocortex than wild-type littermate controls (23.9 ± 0.5 versus 26.4 ± 0.4; $p < 0.005$, Student's t test, 8 mice per genotype), consistent with synaptic/neuronal degeneration as a result of reduced TGF-β1 expression.

Lack of TGF-β1 Expression Results in Changes in Extracellular Matrix Expression in the Brain

TGF-β1 is an important regulator of extracellular matrix synthesis (McCartney-Francis and Wahl, 1994), and we have previously shown that overproduction of TGF-β1 in astrocytes of transgenic mice results in increased production of laminin and fibronectin (Wyss-Coray et al., 1995). Laminin in particular has been implicated in CNS development, neuronal survival, and learning and

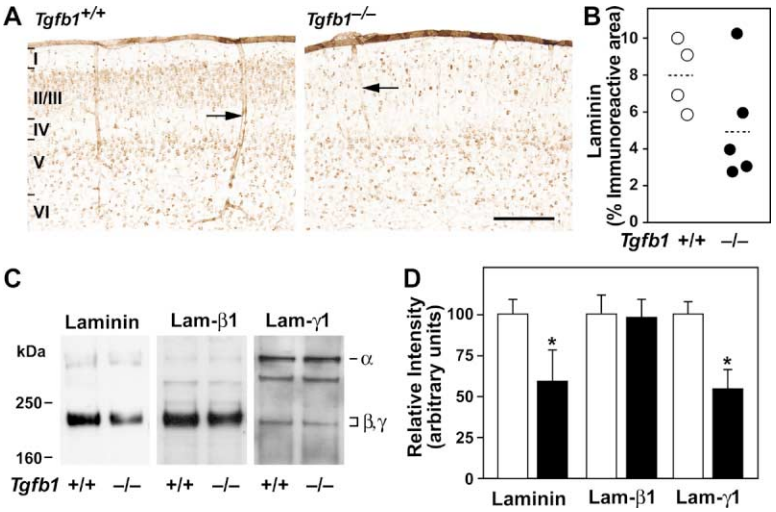

Figure 5. Lack of TGF-β1 Results in Decreased Expression of Laminin in the CNS

(A and B) Sagittal brain sections of unmanipulated P21 *Tgfb1*$^{+/+}$ and *Tgfb1*$^{-/-}$ mice labeled with a panspecific antibody against laminin. Note the decrease in staining in neocortical layers II and III as well as in blood vessels (arrows) in *Tgfb1*$^{-/-}$ compared with *Tgfb1*$^{+/+}$ mice (A). Quantitative analysis of laminin immunostaining in layers II/III in four fields per section and three sections per mouse (B) showed that several *Tgfb1*$^{-/-}$ mice expressed clearly less laminin, although variability is considerable. Each symbol represents one mouse. Scale bar, 150 μm (A).

(C and D) Western blots of hippocampi and cortices from 1- to 3-day-old *Tgfb1*$^{+/+}$ (white bars) and *Tgfb1*$^{-/-}$ mice (black bars) probed with a panspecific antibody against laminin or antibodies against the β1 chain (Lam-β1) or γ1 chain (Lam-γ1) (C). α, β, and γ labels indicate the approximate size of the three laminin chains. Signal intensities were quantified (D) and normalized against actin signals. Values are means ± SEM, *$p < 0.05$, Student's t test, 4–5 mice per genotype.

memory (Luckenbill-Edds, 1997; Venstrom and Reichardt, 1993), and it is conceivable that TGF-β1-dependent changes in extracellular matrix expression may underlie at least some of the degenerative changes observed in TGF-β1-deficient mice. Indeed, laminin expression was reduced in *Tgfb1*$^{-/-}$ mice compared with control littermates (Figure 5). Immunostaining with a panspecific laminin antibody was consistently weaker in medium-size blood vessels of 3-week-old mice lacking TGF-β1 (Figure 5A). In addition, there was a 40% reduction in laminin immunoreactivity in neocortical layers II and III, although this did not reach significance due to the heterogeneity among *Tgfb1*$^{-/-}$ mice (Figures 5A and 5B). Using the same panspecific antibody, laminin protein expression was significantly reduced by Western blot in hippocampi and cortices from 1- to 3-day-old *Tgfb1*$^{-/-}$ mice. Laminins are heterotrimeric proteins composed of an α, β, and γ chain (Luckenbill-Edds, 1997). Each chain exists in different isoforms, forming at least 12 different laminins. In *Tgfb1*$^{-/-}$ mice, the laminin γ1 chain was reduced in addition to total laminin, whereas laminin β1 levels were unchanged (Figures 5C and 5D). These results indicate that TGF-β1 is necessary for normal laminin expression in the CNS.

Primary Neurons from TGF-β1-Deficient Mice Show Reduced Survival that Is Not Rescued by Exogenous Sources of TGF-β1

Primary hippocampal/cortical neurons from *Tgfb1*$^{-/-}$ mice showed reduced neuronal cell numbers after 5 and 11 days in culture compared to primary neurons from *Tgfb1*$^{+/+}$ mice (Figures 6A and 6B), consistent with the observations in *Tgfb1*$^{-/-}$ mice. Neurons from *Tgfb1*$^{-/+}$ mice showed intermediate survival. In contrast, glial cell numbers, which made up around 5% of cultured cells, were not significantly different in cultures from *Tgfb1*$^{-/-}$ and *Tgfb1*$^{+/+}$ mice (Figure 6B). Interestingly, addition of recombinant TGF-β1, both bioactive peptide or the latent form, which consists of active peptide plus propeptide (Munger et al., 1997), failed to increase survival of *Tgfb1*$^{-/-}$ neurons (Figure 6C) at concentrations that consistently result in prominent gene activation and were shown to be optimal to promote survival of wild-type neurons in culture (Chalazonitis et al., 1992). In our hands, recombinant TGF-β1 did not increase survival of wild-type neurons, which may be due to the type of neurons and the culture conditions used (e.g., addition of B27 supplement). Similarly, culturing primary *Tgfb1*$^{-/-}$ or *Tgfb1*$^{+/+}$ neurons with primary astrocytes from *Tgfb1*$^{-/-}$ or *Tgfb1*$^{+/+}$ mice failed to increase survival in a TGF-β1-dependent way (Figure 6C). In contrast, survival of both wild-type and *Tgfb1*$^{-/-}$ neurons is significantly increased by astrocyte-derived factors independent of the TGF-β1 genotype. This failure of exogenous TGF-β1 to increase cell survival in *Tgfb1*$^{-/-}$ neurons could have several reasons, including a permanent defect in these cells as a result of TGF-β1 deficiency during development, a requirement for autocrine TGF-β1 signaling, or a requirement for TGF-β1 release and signaling at synapses (Chin et al., 2002; Zhang et al., 1997).

Prominent Microgliosis in *Tgfb1*$^{-/-}$ Mice Does Not Involve Specific Immune-Mediated Mechanisms

We observed that brain regions with caspase- or TUNEL-positive neurons in KA-injected *Tgfb1*$^{-/+}$ adult mice contained F4/80-positive activated microglia (Figures 7C and 7D), indicating an inflammatory response in areas of degenerating neurons. KA-injected adult *Tgfb1*$^{+/+}$ mice on the NIH background, which showed no or very few caspase-positive cells (Figures 2B and 7A), did not display F4/80-positive activated microglia (Figure 7B).

Microgliosis was even more prominent in mice completely lacking TGF-β1. Unmanipulated *Tgfb1*$^{-/-}$ mice showed a striking microgliosis in the neocortex and hippocampus at P1 and even more so at P21 (Figures 7F and 7H). At P21, TGF-β1 expression in *Tgfb1*$^{-/+}$ mice was sufficient to prevent microgliosis, and the area of F4/80-positive cells in the cortex was not significantly different from *Tgfb1*$^{+/+}$ mice (Figure 7H). Interestingly, no concomitant increase in astrocyte activation was ob-

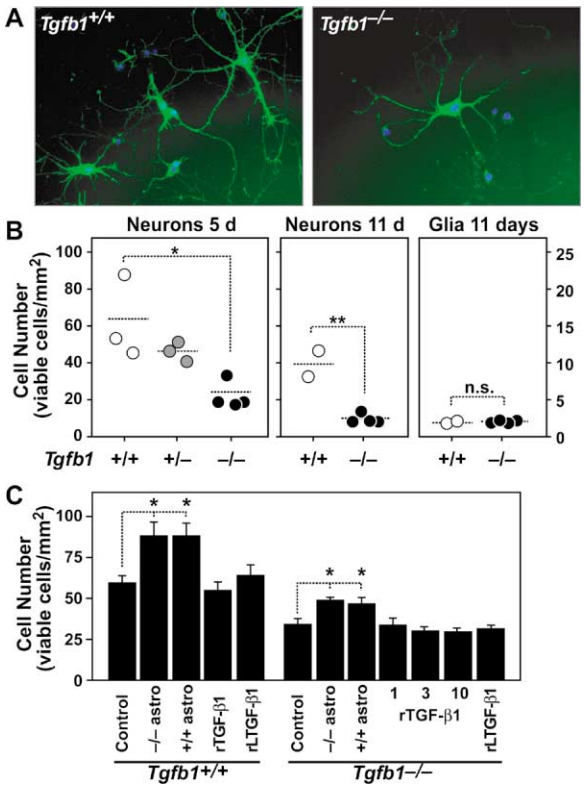

Figure 6. TGF-β1-Deficient Primary Neurons Show Reduced Survival in Culture

(A) Representative examples of *Tgfb1*$^{+/+}$ and *Tgfb1*$^{-/-}$ primary neurons stained for F-actin with BODIPY-FL-Fallacidin (green) and for DNA with Hoechst (blue) after 11 days in culture.

(B) Primary neuronal cultures were stained as above at the indicated time points, and the number of viable neurons per mm^2 was counted based on the absence of pyknotic nuclei. *Tgfb1*$^{-/-}$ cultures had significantly fewer viable neurons than *Tgfb1*$^{+/+}$ controls (*$p < 0.05$, Tukey Kramer; **$p < 0.005$, Student's t test), but the number of glial cells was not different (Student's t test). Each symbol represents the mean value from three individual cultures and eight to nine fields per culture established from one mouse. Note the different scale for neurons and glial cells.

(C) Exogenous recombinant human TGF-β1 (rTGF-β1; 1 ng/ml or as indicated) or recombinant human latent TGF-β1 (rLTGF-β1; 1 ng/ml) could not rescue neuron survival in *Tgfb1*$^{-/-}$ cultures. Cell numbers were counted as above after 5 days in culture. Neurons were also cocultured with wild-type or *Tgfb1*$^{-/-}$ primary astrocytes (astro) in transwell plates. Values are means ± SEM, *$p < 0.05$ compared with controls for the same genotype, Tukey-Kramer test, 3–4 mice per genotype.

served in *Tgfb1*$^{-/-}$ compared with *Tgfb1*$^{+/+}$ mice (Figures 7N and 7O). Because *Tgfb1*$^{-/-}$ mice succumb to a lethal systemic autoinflammation characterized by infiltration of CD4-positive lymphocytes into various peripheral organs at the time of weaning around 3 weeks of age (Geiser et al., 1993; Kulkarni et al., 1993; Shull et al., 1992) (Figure 7M), it was possible that the microgliosis and neurodegeneration we observed were the result of a specific immune reaction in the brain. However, we found, on average, less than ten CD4-positive lymphocytes per brain section in P21 *Tgfb1*$^{-/-}$ mice (Figure 7K). More importantly, *Tgfb1*$^{-/-}$ mice deficient in the recombination associated gene (*Rag1*$^{-/-}$) and, thus, lacking functional B and T lymphocytes showed a similar intensity and pattern of TUNEL staining (Figures 7P and 7Q) and microglial activation (Figures 7F and 7G) as immunocompetent *Tgfb1*$^{-/-}$, *Rag1*$^{+/+}$ mice. Whereas peripheral tissues including heart (Figure 7M), lung, and liver (data not shown) were severely inflamed in *Tgfb1*$^{-/-}$ mice, no lymphocyte and CD4 cell infiltration was detected in the heart (Figure 7L), brain, or other peripheral organs (data not shown) of a *Tgfb1*$^{-/-}$, *Rag1*$^{-/-}$ mouse. Therefore, neurodegeneration and microgliosis in *Tgfb1*$^{-/-}$ mice appear to be independent of lymphocyte-mediated effects.

Discussion

The use of mice lacking or overexpressing TGF-β1 in the CNS has allowed us to demonstrate that this growth factor plays a critical role in maintaining neuronal integrity and survival and in regulating microglial activation. At least some of these effects may be the result of reduced extracellular matrix production. Despite their sharing of the same receptor with TGF-β1, the isoforms TGF-β2 and TGF-β3 could not substitute for deficiency in TGF-β1 expression.

The current results are consistent with TGF-β1 increasing the probability of neuronal survival rather than being an absolute requirement for survival. During postnatal development, loss of TGF-β1 increases neuronal cell death most prominently at P1 when neurogenesis is highest, and this is still ongoing at high levels in P21 *Tgfb1*$^{-/-}$ mice but not in wild-type littermates (Figure 4). Consistent with these findings, primary neurons lacking TGF-β1 showed reduced survival in culture compared with wild-type controls (Figure 6). In addition, TGF-β1 deficiency in adult *Tgfb1*$^{-/+}$ mice does not result in overt neurodegeneration but increases neuronal cell loss after excitotoxic injury (Figure 2) and promotes age-related loss of synaptophysin immunoreactivity (see Results). At P21, unmanipulated *Tgfb1*$^{-/-}$ mice exhibit reduced synaptic density compared to *Tgfb1*$^{+/+}$ littermate controls, and the loss of synaptophysin immunoreactivity in P21 mice of all genotypes correlates inversely with the amount of TGF-β1 mRNA expression in the neocortex (Figure 4). Together, these results demonstrate that TGF-β1 is a survival factor for mouse CNS neurons in vivo and in cell culture.

Interestingly, exogenous TGF-β1 was not able to prevent the accelerated cell death in cultured *Tgfb1*$^{-/-}$ neurons. Thus, addition of recombinant TGF-β1 at high concentrations or coculture with primary astrocytes failed to increase survival of cultured *Tgfb1*$^{-/-}$ neurons in a TGF-β1-dependent way (Figure 6). In contrast, astroglial overproduction of TGF-β1 in wild-type mice is sufficient to increase the survival of neurons to acute and chronic injury (Figure 1). Thus, the lack of TGF-β1 during early development cannot be fully compensated for by supplying TGF-β1 in culture, but astroglial-derived TGF-β1 is sufficient to increase neuronal survival after injury in the adult brain of wild-type mice. Alternatively, neurons may need to produce their own TGF-β1 in an autocrine fashion to overcome the lack of TGF-β1 during development. Such a critical action of TGF-βs in neuronal development is supported by a recent study using rat mes-

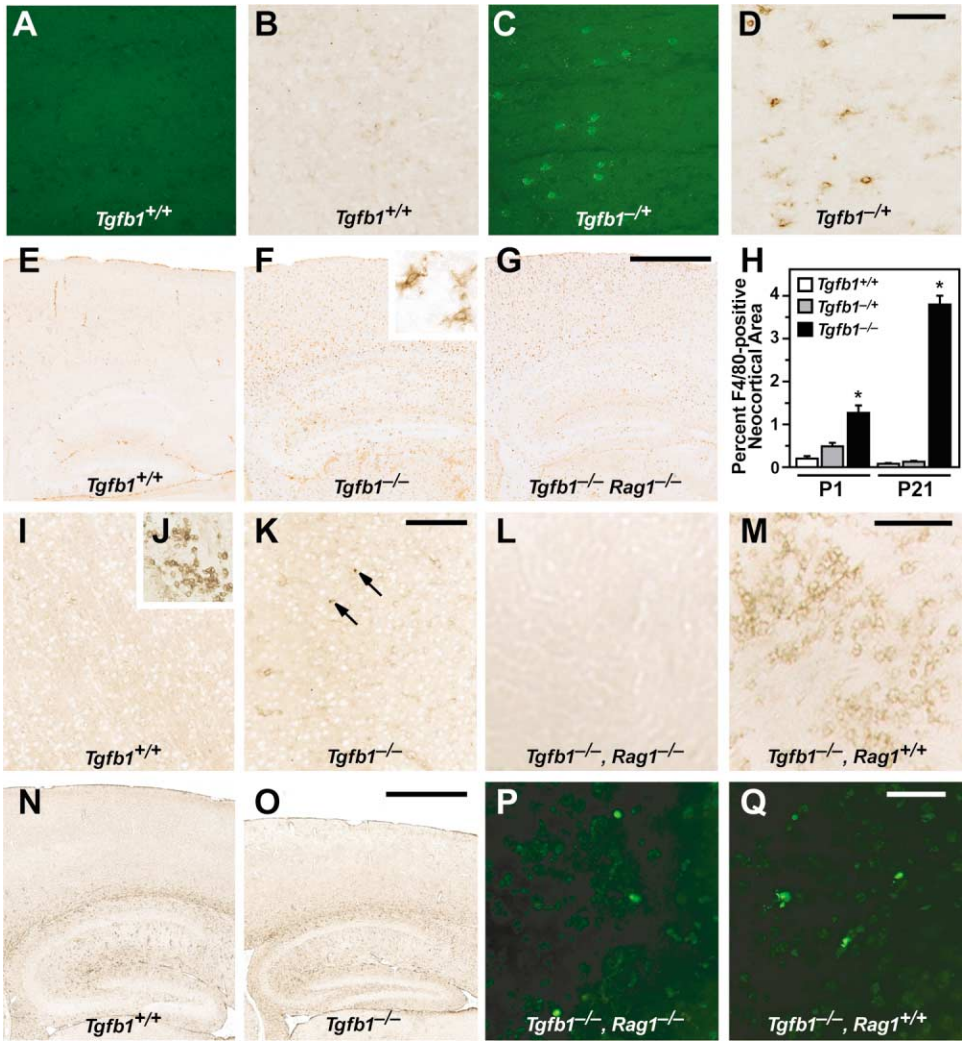

Figure 7. Microglial Activation and Neuronal Cell Death in *Tgfb1*$^{-/-}$ Mice Is Independent of Specific Immune Mechanisms

(A–D) Caspase-positive cortical cells in KA-injected adult *Tgfb1*$^{-/+}$ mice are associated with microglial activation. Adjacent coronal cortical brain sections were labeled for activated caspases (A and C) or immunolabeled for the microglial marker F4/80 (B and D).

(E–G) F4/80 immunolabeling shows extensive microgliosis in P21 *Tgfb1*$^{-/-}$ mice (F) that was independent of specific immune responses as demonstrated in lymphocyte-deficient *Tgfb1*$^{-/-}$, *Rag1*$^{-/-}$ mice (G).

(H) The percent neocortical area occupied by F4/80-positive microglia was significantly larger in *Tgfb1*$^{-/-}$ than in *Tgfb1*$^{-/+}$ or *Tgfb1*$^{+/+}$ littermate control mice at P1 and P21. Values are mean ± SEM, *$p < 0.01$, Tukey-Kramer test, 3–5 mice per genotype.

(I–K) Very few CD4-positive lymphocytes (arrows in [K]) were detected in brain sections of *Tgfb1*$^{-/-}$ mice immunolabeled for CD4 at P21. A mouse with EAE (J), which served as a positive control, showed extensive infiltration of CD4 cells into the periventricular region.

(L and M) Extensive CD4 cell infiltration in the heart of a P21 *Tgfb1*$^{-/-}$, *Rag1*$^{+/+}$ mouse (M), whereas a lymphocyte-deficient *Tgfb1*$^{-/-}$, *Rag1*$^{-/-}$ heart has no infiltrates (L).

(N and O) GFAP immunostaining was not altered by lack of TGF-β1 at P21.

(P and Q) Lymphocyte-deficient *Tgfb1*$^{-/-}$, *Rag1*$^{-/-}$ mice (P) show similar numbers and pattern of TUNEL-positive cells as *Tgfb1*$^{-/-}$, *Rag1*$^{+/+}$ mice (Q).

Scale bars, 50 μm (A–D, P, and Q), 750 μm (E–G, N, and O), and 100 μm (I, K, L, and M).

encephalic cultures and chick embryos to show a requirement for TGF-βs in the development and survival of dopaminergic neurons (Farkas et al., 2003). Interestingly, similar developmental mechanisms may be at play in peripheral organs. Transgenic overproduction of TGF-β1 from the liver could not overcome the lethal phenotype of *Tgfb1*$^{-/-}$ mice, despite restoration of serum TGF-β1 to wild-type levels (Longenecker et al., 2002), and adenoviral overproduction of TGF-β1 in pregnant *Tgfb1*$^{-/+}$ mice, and consequently in embryos, failed to rescue *Tgfb1*$^{-/-}$ embryos from lethality (Chagraoui et al., 2001). Last, TGF-β1 may have to be produced and released at the synapse to increase survival of *Tgfb1*$^{-/-}$ neurons or rescue the loss in synaptic density in TGF-β1-deficient mice. This possibility is supported by recent studies implicating TGF-β1 or the TGF-β signaling pathway in synaptic growth and function in *Aplysia* (Chin et al., 2002; Zhang et al., 1997) and *Drosophila* (Sweeney and Davis, 2002; Zheng et al., 2003). Studies using conditional gene knockout strategies to interfere with neu-

ronal TGF-β signaling in adult mice could help in assessing the importance of TGF-β signaling in synaptic growth and function in the mammalian CNS.

Different mechanisms have been postulated to explain how TGF-β1 protects neurons against injury and degeneration (Flanders et al., 1998), and because neurons as well as glial cells can express TGF-β receptors (Flanders et al., 1998), neuronal protection could be direct or indirect. Our studies here show that TGF-β1 deficiency results in abnormal expression and reduction of the extracellular matrix protein laminin, which might be responsible, at least in part, for the reduced survival and increased susceptibility of neurons to injury. Laminin has been implicated in CNS development, neuronal survival, and learning and memory (Luckenbill-Edds, 1997; Venstrom and Reichardt, 1993). TGF-β1 is a known inducer of laminin synthesis in cultured cells and in the CNS of transgenic mice (Wyss-Coray et al., 1995), and the current results indicate that TGF-β1 is also required for normal expression of laminin in the brain (Figure 5). In addition, degradation of laminin, specifically laminin 10 (Indyk et al., 2003), by tissue plasminogen activator (tPA) and/or plasmin has been proposed to be responsible for neuronal cell death associated with KA-induced excitotoxicity (Chen and Strickland, 1997). This degradation is presumed to eliminate the critical surface for support of neuronal function and survival (Luckenbill-Edds, 1997; Venstrom and Reichardt, 1993). Because TGF-β1 is a key inducer of plasminogen activator inhibitor (PAI) 1 (Loskutoff et al., 1989), which is probably the main inhibitor of tPA (Loskutoff et al., 1989) and thus the above KA-mediated cascade (Tsirka et al., 1996), neuronal survival in *Tgfb1*$^{-/-}$ mice may be reduced due to accelerated degradation of laminin.

TGF-β1 may also promote neuronal survival by interacting with neurotrophins. Although it is considered not to be a prototypic neurotrophic factor for purified neurons (Chalazonitis et al., 1992; Unsicker and Krieglstein, 2000, 2002), TGF-β1 has been shown to synergize with neurotrophins or to be required to mediate at least some of the effects of NGF, BDNF, NT-3, and NT4 (reviewed in Unsicker and Krieglstein, 2000, 2002) as well as FGF-2 (Krieglstein et al., 1998) and GDNF (Schober et al., 1999). Interestingly, deficiency in most of these classical neurotrophic factors did not result in overt neurodegeneration in the CNS of mutant mice (Huang and Reichardt, 2001). However, neurotrophin receptor TrkB-deficient mice showed an increase in postnatal apoptosis of CNS neurons and increased susceptibility to injury (Alcantara et al., 1997; Minichiello and Klein, 1996), and postnatal deletion of the *trkB* gene in pyramidal neurons resulted in progressive loss of dendritic arborization and cortical neurodegeneration in mice (Xu et al., 2000). Deficiency of the TrkB ligand BDNF resulted in a prominent reduction of calbindin and parvalbumin expression (Jones et al., 1994), and mice lacking TrkB and TrkC receptors show massive cell death of hippocampal and cerebellar granule cells (Minichiello and Klein, 1996). The phenotype observed in the current study appears somewhat similar to the one associated with TrkB signaling deficiency in TrkB knockout mice. Interestingly, treatment of primary rat cortical neurons with TGF-β1 increased the expression of TrkB and its ligand BDNF, whereas blocking TrkB signaling abolished TGF-β1's neuroprotective effects (Sometani et al., 2001). Thus, it is conceivable that a deficiency in TrkB-dependent neurotrophin signaling contributes to the neuronal defects observed in *Tgfb1*$^{-/-}$ mice.

Whether and how the effects of TGF-β1 deficiency on neurons relate to the microgliosis in *Tgfb1*$^{-/-}$ is unclear. TGF-β1 is a key regulator of specific and innate immune functions (Dennler et al., 2002; Letterio and Roberts, 1997, 1998). It can suppress specific immune responses by inhibiting T lymphocyte proliferation and maturation or by inducing apoptosis (Letterio and Roberts, 1998), and it reduces MHC class II expression (Letterio and Roberts, 1998). Genetic studies indicate that uncontrolled MHC class II expression is the cause of lymphocyte-mediated autoimmune reactions and the wasting syndrome in *Tgfb1*$^{-/-}$ mice (Diebold et al., 1995; Geiser et al., 1993; Letterio et al., 1996). Our studies with *Rag1*$^{-/-}$ mice, which lack mature T and B lymphocytes and fail to mount an antigen-specific immune response (Mombaerts et al., 1992), demonstrate that the accumulation of apoptotic neurons and neuroinflammation are not caused by these immune cells (Figure 7).

However, besides these effects on specific immune mechanisms, TGF-β1 regulates innate immune functions, including the differentiation and proliferation of macrophages (Letterio et al., 1996), which are closely related to microglia. In this role, TGF-β1 has been shown to facilitate effective corpse clearance or phagocytosis of apoptotic cells by macrophages and other cells (Fadok et al., 1998, 2000; Freire-de-Lima et al., 2000), and it promotes the clearance of amyloid peptides by cultured microglia (Wyss-Coray et al., 2001). Hence, mice lacking TGF-β1 may be less efficient in the clearance of randomly dying cells or in suppressing inflammation around active phagocytes. Interestingly, cells undergoing cell death have been shown to secrete TGF-β1, possibly to reduce local inflammation and prevent degeneration of additional surrounding cells (Chen et al., 2001). Although our study cannot establish a cause-effect relationship between microgliosis and apoptotic changes in neurons, it is intriguing that microgliosis is absent in adult *Tgfb1*$^{-/+}$ mice, but activated microglia are closely associated with apoptotic neurons in the same mice after injury (Figure 7). Similarly, P1 *Tgfb1*$^{-/-}$ mice showed apoptotic cells surrounded by activated microglia in various brain regions. Therefore, it could be argued that neurons and other cells exposed to proapoptotic signals or normal stress may be cleared less efficiently by microglia in TGF-β1-deficient mice, thus contributing to "spreading" of cell death or degeneration and inflammation from focal points of injury.

Our results show that TGF-β1 has a nonredundant function in supporting neuronal survival and integrity and in regulating microglial activation. Lack of TGF-β1 exacerbates neuronal injury during development or after excitotoxic lesions, possibly due to reduced trophic support and impaired microglial responses. It is important to note that genetic polymorphisms in the human *TGFB1* gene are associated with different levels of TGF-β1 in the serum (Grainger et al., 1999) and possibly in other tissues as well. Interestingly, increased cerebral TGF-β1 production reduced β-amyloid accumulation in the brain parenchyma of mice that model AD (Wyss-Coray et al., 2001), and cortical TGF-β1 mRNA levels in

human AD cases correlated inversely with the extent of β-amyloid deposition in the midfrontal cortex (Wyss-Coray et al., 2001). Conceivably, individuals with lower levels of TGF-β1 production may be more susceptible to amyloidosis and neurodegeneration.

Experimental Procedures

Mice

TGF-β1 transgenic mice expressing porcine TGF-β1 under control of the glial fibrillary acidic protein (GFAP) promoter in astrocytes at low or medium levels (Wyss-Coray et al., 1995, 2000) were used on the C57BL/6J genetic background. All transgenic mice used in the study were heterozygous for the TGF-β1 transgenes. *Apoe*$^{-/-}$ mice (C57BL/6J-Apoe^{tm1Unc}) were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice lacking one (*Tgfb1*$^{-/+}$) or both (*Tgfb1*$^{-/-}$) copies of the *Tgfb1* gene have been described (Bonyadi et al., 1997) and were obtained from Dr. R. Akhurst (University of California, San Francisco). On the NIH/Ola background, *Tgfb1*$^{-/+}$ mice show no gross abnormalities, and more than 80% of *Tgfb1*$^{-/-}$ conceptuses reach parturition. However, *Tgfb1*$^{-/-}$ mice die shortly after 3 weeks of age of a severe multifocal autoinflammatory disorder (Shull et al., 1992) and, as a result, were obtained via *Tgfb1*$^{-/+}$ × *Tgfb1*$^{-/+}$ crosses. *Tgfb1*$^{-/+}$ mice on the C57BL/6J genetic background (Shull et al., 1992) were obtained from Jackson Laboratories. *Rag1*$^{-/-}$ mice (Balb/c background) were obtained from Dr. R. Locksley (University of California, San Francisco) and crossed with *Tgfb1*$^{-/+}$ mice for two generations. Only 2 out of 64 mice (expected frequency 16 out of 64) were born from crossing *Tgfb1*$^{-/+}$, *Rag1*$^{-/-}$ with *Tgfb1*$^{-/+}$, *Rag1*$^{-/-}$ mice. This lethality was most likely due to the Balb/c genetic background of the *Rag1*$^{-/-}$ breeders which is associated with embryonic lethality in *Tgfb1*$^{-/-}$ mice. Brain tissue from a mouse with severe experimental autoimmune encephalomyelitis (EAE) was kindly provided by Dr. E. Foehr (Gladstone Institutes, San Francisco). Mice were genotyped by subjecting proteinase K-digested tail tissue to touchdown PCR (Hecker and Roux, 1996) and were kept under a 12 hr light/dark cycle with free access to sterile water and food (Picolab Rodent Diet 20, #5053, PMI Nutrition International). Animal care was in accordance with institutional guidelines.

Excitotoxic Injury Model

Kainic acid (KA) crosses the blood-brain barrier and induces excitotoxic CNS injury, particularly in the hippocampus and the neocortex (Schauwecker and Steward, 1997). KA (Sigma) was dissolved in PBS and injected intraperitoneally in one dose at 15 mg/kg (mice on NIH/Ola or NIH/Ola × Balb/c mixed background) or 25 mg/kg (C57BL/6J mice). Seizure activity was scored from 0 to 5, with 0 showing no behavioral changes and 5 showing constant rearing and falling as described (Schauwecker and Steward, 1997). All KA-injected mice reached at least stage 3. Control mice were injected with PBS. Mice were killed 24 hr after the injection, except for microtubule-associated protein 2 (MAP-2) and synaptophysin immunolabeling experiments, for which mice were killed 5 days after injection.

Tissue Preparation

Mice were anesthetized with 400 mg/kg chloral hydrate (Sigma) and flush perfused transcardially with 0.9% saline. Brains were removed and snap-frozen in isopentane for extraction of RNA or embedded in Tissue-Tek OCT compound (Sakura) and cut into 12 μm thick sections with a cryostat. For some experiments, hemibrains cut sagitally were immersion fixed in freshly prepared phosphate-buffered 4% paraformaldehyde (PFA) (pH 7.4) at 4°C for 48 hr and cut into 40 μm thick sections with a vibratome (Leica) or sunk through 30% sucrose in PBS and cut into 30 μm thick sections with a cryotome (Leica). For most histological analyses, mice were perfused with saline, then with PFA, and postfixed for 48 hr in PFA. Fixed brains were embedded in paraffin and cut into 6 μm thick sections.

Histochemistry

For hematoxylin and eosin staining, sections were deparaffinized, washed in water, stained in Mayer's hematoxylin solution (Sigma) for 15 min, washed in water, placed in 80% ethanol for 2 min, counterstained with eosin-phloxine solution (Sigma) for 2 min, dehydrated, and mounted. For cresyl violet staining, sections were deparaffinized, washed in PBS, stained in 0.02% cresyl violet (Sigma) in acetate buffer for 40 min, washed in 95% ethanol plus acetic acid, dehydrated, and mounted.

Immunohistochemistry

Sections were pretreated for 20 min in Tris-buffered saline with 0.05% Tween 20 (TBST) with 0.1% Triton X-100 and 0.3% H_2O_2 (Sigma) to maximize antigen exposure and quench endogenous peroxidase activity, respectively. Cryosections were fixed in 4% PFA for 20 min or in acetone for 4 min (for CD4 and F4/80 labeling). To block nonspecific interactions, all sections were incubated for 5–7 min in Superblock (Scytek) or for 1 hr in 10% serum (Vector) of the species in which the secondary antibody was raised. Sections were then incubated with primary antibodies against MAP-2 (Roche, dilution 1:1000), synaptophysin (Roche, 1:800), calbindin (Sigma, 1:2500), neuronal nuclear protein (NeuN; Chemicon, 1:1000), the neuronal differentiation and migration marker doublecortin (Chemicon, 1:15,000), proliferating cell nuclear antigen (PCNA; DAKO, 1:1500), neurofilament (Sternberger Monoclonals, 1:5000), cleaved caspase 3 (Cell Signaling, 1:1500), laminin (Sigma, 1:100), the macrophage/microglial marker F4/80 (Serotec, 1:100), the astrocytic marker GFAP (DAKO, 1:1000), or the helper T cell marker CD4 (BD Pharmingen, 1:60) at 4°C overnight.

For light microscopy, sections were incubated with a biotinylated secondary antibody (Vector; 1:200) for 1 hr and developed with diaminobenzidine and H_2O_2 (Sigma) using the ABC Elite kit (Vector). For fluorescence microscopy, sections were incubated with fluorescein isothiocyanate- (FITC) or Cy5-conjugated secondary antibodies (Vector or Jackson Immunoresearch, respectively, 1:200) for 1 hr, mounted with Vectashield (Vector), and viewed with a Radiance 2000 laser scanning confocal system (Bio-Rad) mounted on an Olympus BX-60 microscope.

MAP-2 and synaptophysin are reliable indicators of, respectively, synaptic and dendritic damage and have been demonstrated to correlate well with loss of cognitive function in murine models of neurodegeneration and humans with AD (Buttini et al., 1998; Terry et al., 1991). The percent area of neuropil occupied by MAP-2-immunoreactive processes and synaptophysin-immunoreactive presynaptic terminals was determined by confocal microscopy and computer-aided image analysis as described (Masliah et al., 1992a, 1992b). F4/80-labeled sections were imaged with an Olympus UTV-1X digital camera mounted on an Olympus BX-60 microscope. The percent cortical area covered by F4/80 labeling in six microscopic fields (10× objective) in four to six sections per brain was quantified using the Bioquant 98 software (R&M Biometrics).

For calbindin quantification, digitized images of immunostained sections were obtained with a video camera mounted on a Zeiss microscope at a final magnification of 300× and then analyzed with the Quantimet 570C system. For each mouse, two sagittal sections (300 μm apart) were selected, and from each section a total of ten random fields (0.16 mm^2) in neocortical cell layers 2–3 and 4–5 were analyzed. The total number of positive cells per layer was averaged and expressed as relative numbers per mm^2.

TUNEL Labeling

DNA fragmentation, a hallmark of apoptosis, was detected by in situ labeling with the terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) assay kit (Roche). Briefly, six to nine cryosections per brain were fixed in 4% PFA for 20 min, permeabilized with 0.1% Triton X-100, acetylated for 10 min, dehydrated through ethanol series, incubated with digoxygenin-labeled dUTP and deoxynucleotidyl transferase at 37°C for 1 hr, and labeled with a FITC- or Rhodamin-conjugated anti-digoxygenin antibody (Roche). TUNEL-positive cells were viewed and counted by fluorescence microscopy. For double labeling, sections were either stained post-TUNEL with NeuroTrace following the manufacturer's directions (Molecular Probes, 1:200) or coincubated with NeuN or neurofilament antibodies as described and detected with Alexa 647-conjugated secondary antibodies (Molecular Probes, 1:200).

Activated Caspase Labeling

Activated caspases were detected with the fluorescently labeled peptidic inhibitor CaspACE FITC-VAD-FMK (Promega), which

crosses the plasma membrane and binds to all activated caspases in the cell. Cryosections were thawed, pretreated with 0.1% Triton X-100 (Sigma) in TBST for 20 min, washed 3 × 5 min in TBST, and incubated with CaspACE FITC-VAD-FMK (20 μM) for 1 hr in the dark. They were then washed 5 × 5 min in TBST and fixed in 4% PFA for 30 min, washed 5 × 5 min in TBST, quickly rinsed in dH_2O, and mounted with Vectashield (Vector). Caspase-positive cells were viewed and counted by fluorescence microscopy by an observer blinded with respect to the genotype of the sections. Specific activated caspase 3 labeling is described under immunohistochemistry.

RNA Extraction and Analysis

Total RNA was isolated from tissues with TRI-Reagent (Molecular Research Center) or Tripure (Boehringer Mannheim). RNA was analyzed by solution hybridization Rnase Protection Assay with antisense riboprobes complementary to TGF-β1 or actin mRNA (Wyss-Coray et al., 2000) or by real-time PCR using an ABI-Prism-7700 sequence detector and following the manufacturer's instructions (ABI).

Postnatal Primary Neuronal and Astroglial Cell Cultures

Mixed hippocampal-neocortical primary neuronal cultures were prepared from P1 mice of matings between heterozygous *Tgfb1*$^{-/+}$ mice on the NIH background as described previously (Tesseur et al., 2000). Briefly, single-cell suspensions obtained from both hippocampi and cortices of individual pups were plated separately on poly-L-lysine-coated 12-well plates at a density of 5 × 10^4 cells/well in minimal essential medium (MEM) supplemented with 10% horse serum and subsequently cultured in serum-free Neurobasal A medium supplemented with B27 (GIBCO, BRL). Cytosine-β-arabinofuranoside (5 μM) was added 24 hr after plating to prevent/restrict glial cell proliferation. For primary astrocytes, mice of the above breeding were used at P3 (Tesseur et al., 2000).

For some experiments, neurons were cocultured with *Tgfb1*$^{-/-}$ or *Tgfb1*$^{+/+}$ astrocytes seeded into transwell tissue culture inserts (Costar, Corning) during the entire time of culture. The membranes (0.4 μm) will allow factors to diffuse between the neuronal and astrocyte compartments but will not allow cells to transmigrate. At the end of the experiment, astrocytes were discarded and neurons were stained as described below. If indicated, recombinant human TGF-β1 or recombinant human latent TGF-β1 (both R&D systems) were added to neurons throughout the time of culture at the indicated concentrations every other day.

Primary neurons were analyzed at 5 or 11 days in culture. After fixation in 4% PFA, cells were labeled with BODIPY-FL-phallacidin (Molecular Probes) according to manufacturer's instructions to stain the actin cytoskeleton or with an antibody to MAP-2 (see above). Cultures were then stained with Hoechst 33342 (Molecular Probes) to label DNA and examined with an Olympus IX70 inverted fluorescence microscope connected to a Coolsnap HQ Photometrics camera by an experimenter blinded toward their genotype. Images were processed and analyzed using Metamorph Imaging software (Universal Imaging Corp.) and Adobe Photoshop.

Protein Extraction and Immunoblotting

Hippocampi and cortices from 1- to 3-day-old *Tgfb1*$^{-/-}$ and *Tgfb1*$^{+/+}$ mice were lysed in extraction buffer consisting of 50 mM HEPES (pH 7.5), 0.5 M NaCl, 1% Triton X-100, 0.125% Tween-20, 0.5% deoxycholate, 1% sodiumdodecylsulphate, and 1 tablet complete proteinase Inhibitor (Roche) per 50 ml extraction buffer. Equal amounts of protein were subjected to SDS-PAGE under reducing conditions in 3%–8% NuPage gels (Novex), transferred to nitrocellulose membranes (Biorad), and probed with antibodies against laminin (Sigma, 1:1000), laminin β1 chain (Chemicon, 1:500), or laminin γ1 chain (Chemicon, 1:1000), followed by peroxidase-conjugated species-specific secondary antibodies. To adjust for loading differences, blots were stripped and reprobed with an antibody against actin (Chemicon, 1:250). Binding of secondary antibodies was visualized by enhanced chemiluminescence (ECL, Amersham Biotech), and signal intensities were quantified with Metamorph Imaging software. Relative laminin expression levels were calculated by normalizing against actin signals.

Statistical Analysis

Differences between two means were assessed by Mann-Whitney U or Student's t test for nonparametric or parametric data, respectively. Differences among multiple means of data with parametric distribution were assessed by ANOVA followed by Tukey-Kramer post hoc test. All statistical analyses were done using the Statview software (SAS Institute).

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