TGF-β1 activates two distinct type I receptors in neurons: implications for neuronal NF-κB signaling

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Transforming growth factor-βs (TGF-βs) are pleiotropic cytokines involved in development and maintenance of the nervous system. In several neural lesion paradigms, TGF-β1 exerts potent neuroprotective effects. Neurons treated with TGF-β1 activated the canonical TGF-β receptor I/activin-like kinase receptor 5 (ALK5) pathway. The transcription factor nuclear factor-κB (NF-κB) plays a fundamental role in neuroprotection. Treatment with TGF-β1 enhanced NF-κB activity in gelshift and reporter gene analyses. However, ectopic expression of a constitutively active ALK5 failed to mimic these effects. ALK1 has been described as an alternative TGF-β receptor in endothelial cells. Interestingly, we detected significant basal expression of ALK1 and its injury-induced up-regulation in neurons. Treatment with TGF-β1 also induced a pronounced increase in downstream Smad1 phosphorylation. Overexpression of a constitutively active ALK1 mimicked the effect of TGF-β1 on NF-κB activation and neuroprotection. Our data suggest that TGF-β1 simultaneously activates two distinct receptor pathways in neurons and that the ALK1 pathway mediates TGF-β1–induced NF-κB survival signaling.

Introduction

Transforming growth factor-βs (TGF-βs) are pleiotropic cytokines implicated in control of cell growth, differentiation, inflammation, and apoptosis. The mammalian TGF-β isoforms 2 and 3 are expressed in neurons and glial cells of the central nervous system, whereas TGF-β1 expression is largely confined to the meninges and choroid plexus (Flanders et al., 1991). Expression of TGF-β2 and -β3 are mainly regulated by hormonal and developmental signals (Flanders et al., 1998). In contrast, TGF-β1 expression and release increases significantly in response to central nervous system lesions, with activated microglia/macrophages being a major source (Lindholm et al., 1992). TGF-β1 has been shown to protect cultured neurons from hypoxic (Prehn et al., 1993a), excitotoxic (Prehn and Krieglstein, 1994), apoptotic (Prehn et al., 1994), metabolic (Krieglstein et al., 1995), and toxic peptide-induced injury (Meucci and Miller, 1996; Prehn et al., 1996; Ren and Flanders, 1996). In vivo, administration of recombinant TGF-β1 or gene delivery of TGF-β1 potently protects animals against brain injury mediated by ischemic (McNeill et al., 1994; Zhu et al., 2002), excitotoxic (Ruocco et al., 1999), and oxidative stress (Henrich-Noack et al., 1996). Moreover, lack of TGF-β1 expression in neonatal Tgfb1$^{-/-}$ mice results in a widespread increase in degenerating neurons and a prominent microgliosis (Brionne et al., 2003).

Neuroprotection by TGF-β1 has been shown to involve the up-regulation of the antiapoptotic Bcl-2 family proteins Bcl-2 and Bcl-xL (Prehn et al., 1994, 1996; Kim et al., 1998). Both are target genes of the transcription factor nuclear factor-κB (NF-κB; Lee et al., 1999; Tamatani et al., 1999; Bui et al., 2001). Indeed, a recent paper has demonstrated that TGF-β1 activates NF-κB in neurons and that neuroprotection elicited by TGF-β1 requires NF-κB activation (Zhu et al., 2004). Of note, these observations sharply contrast with effects of TGF-β1 described in other cell types and organ systems. For example, TGF-β1 has been shown to inhibit NF-κB activation and to trigger cell death in B cells and hepatocytes (Oberhammer et al., 1992; Arsura et al., 1996).

The present work will aid to provide an explanation for this apparent discrepancy. We demonstrate that TGF-β1 is able to activate two distinct TGF-β type I receptors and signal transduction pathways in neurons: the canonical activin-like kinase 5 (ALK5)/Smad2/3 pathway and a novel, ALK1/Smad1/5-regulated pathway. Furthermore, we demonstrate that signaling through ALK1 mediates the activation of the antiapoptotic NF-κB pathway.

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Abbreviations used in this paper: ALK5, activin-like kinase 5; BMP, bone morphogenetic protein; DIV, days in vitro; GFAP, glial acidic fibrillary protein; MCAO, middle cerebral artery occlusion; NF-κB, nuclear factor-κB; NMDA, N-methyl-D-aspartate; NO, nitric oxide; PI, propidium iodide; SNOC, S-nitrosocysteine; TβRI, TGF-β receptor type I; TGF-β, transforming growth factor-β.

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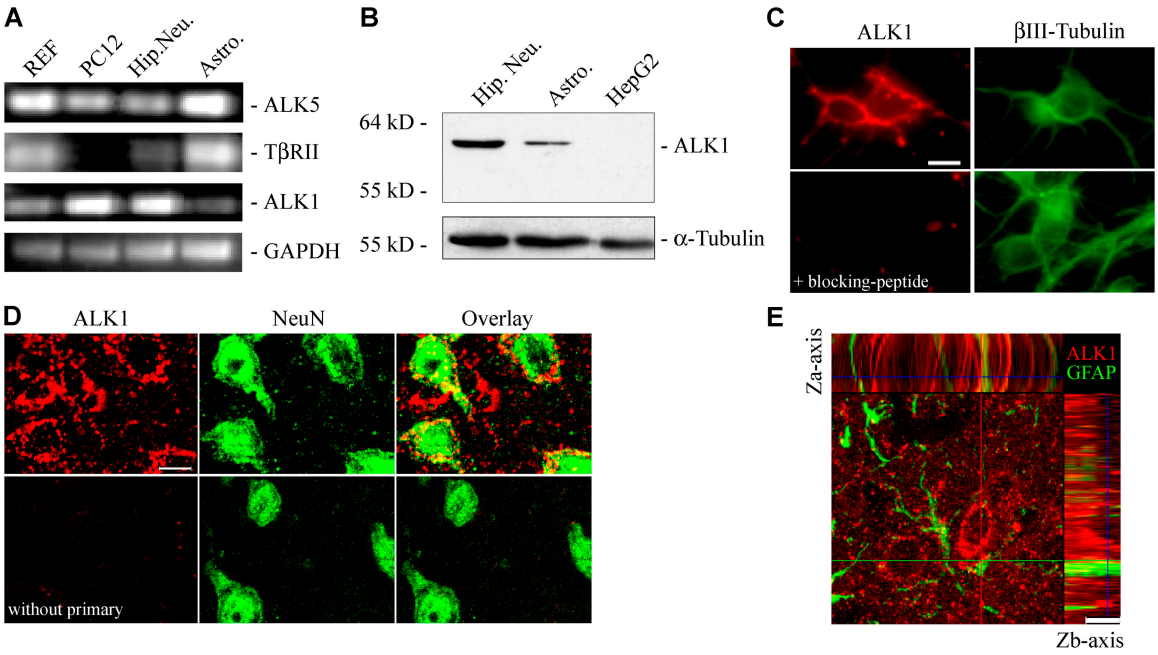

Figure 1. **Neuronal cells express the alternative TGF-β type I receptor ALK1.** (A) Expression of ALK5, TβRII, and ALK1 mRNA was determined by RT-PCR analysis in DIV14 primary cultures of rat hippocampal neurons, secondary cultures of astrocytes, as well as REF and PC12 cell lines. GAPDH expression is shown for normalization purposes. Reaction mixtures without reverse transcriptase served as controls for genomic DNA contamination in all cases (unpublished data). (B) Cell lysates of cultured rat hippocampal neurons, secondary astrocytes, and HepG2 cells were subjected to SDS-PAGE and Western blotting. The membranes were incubated with the ALK1 antibody, which recognized one specific band; α-tubulin band served as loading control. Experiments in duplicate yielded comparable results. (C) Immunofluorescence detection of ALK1 protein in cultured rat hippocampal neurons (red). DIV14 hippocampal cultures were fixed and subsequently incubated with ALK1 antibody. Preincubation of ALK1 antibody with blocking peptide and subsequent immunostaining served as negative control. βIII-Tubulin was used as a neuronal marker (green). (D and E) Immunofluorescence analysis of ALK1 protein expression in coronal sections of the adult rat brain. Representative cortical areas are shown. Slices were double-labeled with antibodies directed against ALK1 protein (red) and neuronal nuclear protein NeuN (D, green) or GFAP (E, green) and visualized by confocal microscopy (D) or confocal stack reconstruction (E). Red and green axes mark the positions of the corresponding perpendicular Z-layers. Bars, 10 μm.

Results

ALK1 receptor is expressed in neurons and the level of expression increases during excitotoxic and ischemic injury

TGF-βs signal through a family of serine/threonine receptor kinases. Binding of TGF-βs to the constitutively active type II receptor initiates the recruitment of type I receptors, which subsequently become phosphorylated and transduce the signals into the cytosol (Shi and Massague, 2003). TGF-β receptor type II (TβRII) and TβRI/ALK5 have been shown to be expressed in the central nervous system and in cultured neurons (Bottner et al., 1996). Recent studies have demonstrated the existence of an alternative TGF-β type I receptor, ALK1 (Lux et al., 1999). To investigate the contribution of different TGF-β receptors in neuronal TGF-β signaling, the expression of ALK5, TβRII, and ALK1 was determined in primary rat hippocampal neurons by RT-PCR analysis. Expression of ALK5 mRNA was detected in the hippocampal neurons as well as in secondary cultures of rat astrocytes, rat PC12 pheochromocytoma cells, and rat embryonic fibroblasts. TβRII was found to be expressed at low levels in hippocampal neurons (Fig. 1 A). Astrocytes and rat embryonic fibroblasts revealed a relatively high expression level, whereas rat pheochromocytoma PC12 cells that do not express TβRII (Lutz et al., 2004) served as a negative control. Interestingly, robust expression levels of ALK1 could be detected specifically in rat hippocampal neurons and PC12 cells; in contrast, ALK1 mRNA levels were markedly lower in astrocytes and fibroblasts (Fig. 1 A). Western blotting experiments using a polyclonal antibody for ALK1 revealed high ALK1 protein expression levels in hippocampal neurons and to a lesser extent in astrocytes. HepG2 hepatocytocellular carcinoma cells, which do not express the ALK1 receptor (Attisano et al., 1993), served as a negative control (Fig. 1 B). Further evidence for neuronal ALK1 expression was revealed by immunofluorescence analysis. Surface-derived immunostaining of ALK1 protein was detected in hippocampal neurons (Fig. 1 C). A specific blocking peptide to the ALK1 antibody abolished the staining. ALK1 immunodetection was also assessed in adult rat coronal brain sections. Hippocampal and cortical neuronal cell bodies positive for the neuronal marker NeuN displayed a substantial ALK1 immunofluorescence. Dendrites and the neuropil revealed a partly punctuated staining as previously described for other TGF-β family receptors (Di Guglielmo et al., 2003; Fig. 1, D and E). Subsequent confocal immunofluorescence analyses, with costaining against glial acidic fibrillary protein (GFAP), revealed that ALK1 expression was largely restricted to neuronal cells (Fig. 1 E).

TGF-β1 has been identified as a growth factor/cytokine up-regulated during brain injury. Therefore, we determined

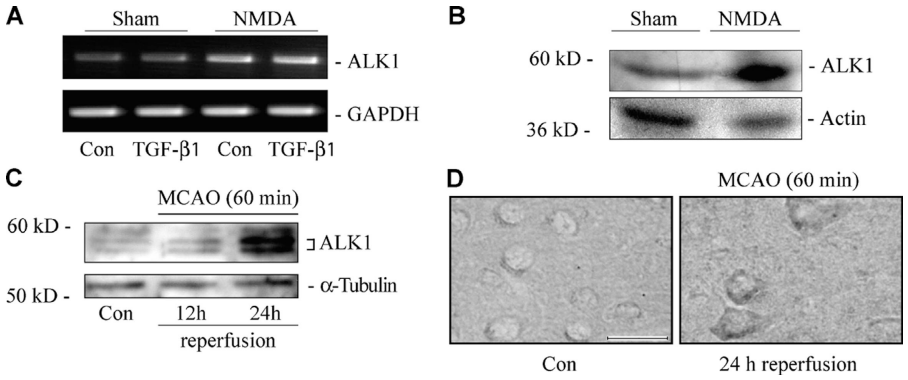

Figure 2. **Injury-induced up-regulation of ALK1 receptor in vitro and in vivo.** Evaluation of ALK1 mRNA (A) and protein levels (B) 24 h after a brief exposure to excitotoxic NMDA (300 μM; 5 min). (A) After the NMDA exposure, cells were treated with 10 ng/ml TGF-β1 or vehicle (2 mg/ml PBS/BSA). 30 cycles of PCR or Western blot for ALK1 receptor expression (B) was performed. GAPDH or actin served as control for equal loading. (C) Western blot analysis of ALK1 protein levels after ischemia/reoxygenation. Lysates were obtained from lesioned cortex and striatum of animals subjected to 1 h of ischemia followed by 12 or 24 h of reoxygenation (MCAO). (D) DAB-immunocytochemistry analysis of MCAO-induced cortical ALK1 expression. 24 h after reoxygenation, lesioned hemispheres were fixed and stained for ALK1 protein. Bar, 20 μm.

whether excitotoxic or ischemic injury also influenced the level of ALK1 mRNA and protein expression. To this end, excitotoxic neuronal injury was induced in hippocampal neurons by a brief exposure to the selective glutamate receptor agonist N-methyl-D-aspartate (NMDA; 300 μM, 5 min). Interestingly, increased mRNA and protein levels of ALK1 were detected 24 h after the NMDA exposure (Fig. 2, A and B). Treatment with TGF-β1 did not have a potentiating effect on ALK1 expression, suggesting that expression of ALK1 is not regulated by TGF-β1. Increased ALK1 protein expression was also observed in the cortical tissue of rats subjected to focal ischemic injury induced by middle cerebral artery occlusion (MCAO; Fig. 2 C). Western blot analysis revealed two specific bands, possibly corresponding to differentially N-glycosylated forms of ALK1 (Lamouille et al., 2002). Cortical brain sections of ischemic rats revealed enhanced neuronal ALK1 staining in comparison with controls (Fig. 2 D), revealing that ALK1, similar to TGF-β1, is up-regulated during brain injury.

TGF-β treatment elicits both ALK5- and ALK1-dependent signaling pathways in primary rat hippocampal neurons

Signaling of TGF-β superfamily members involves ALK-mediated phosphorylation of receptor-regulated Smad proteins. Treatment of hippocampal neurons with TGF-β1 led to transient induction of Smad2 phosphorylation after 30 min, indicative of the canonical, ALK5-initiated, Smad2/3-mediated signal transduction pathway (Fig. 3 A; Nakao et al., 1997). Treatment with bone morphogenetic protein (BMP)-6, which served as a negative control, did not change the status of Smad2 phosphorylation

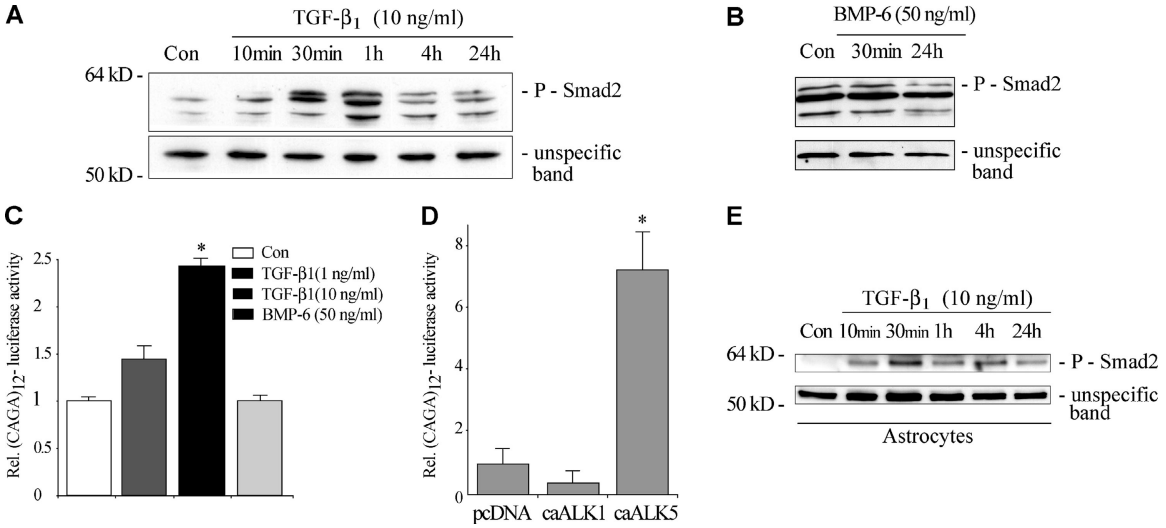

Figure 3. **TGF-β activates the ALK5 signaling pathway in cultured rat hippocampal neurons and astrocytes.** (A) DIV14 rat hippocampal neurons were treated with 10 ng/ml TGF-β1. (B) In parallel experiments, cultures were incubated with 50 ng/ml BMP-6. Controls received vehicle. At the indicated time points, whole cell extracts were obtained in TOTEX buffer and analyzed after Western blotting with a P-Smad2 antibody. An unspecific band served as internal loading control as previously described (Goumans et al., 2002). A duplicate experiment yielded comparable results. (C and D) Dual-luciferase reporter gene analysis for the ALK5 pathway-responsive $(CAGA)_{12}$-luc. 24 h after transfection of DIV6 hippocampal neurons, cells were treated with the indicated cytokines and lysed after 24 h of further incubation. In D, cotransfection was performed with the indicated plasmids. Luciferase responsiveness was normalized to the cotransfected RL-TK-luc. Data are means ± SEM from $n = 4$–6 cultures. *, $P < 0.05$ to control. Experiments were performed in duplicate with similar results. (E) Cultured secondary astrocytes were incubated with 10 ng/ml TGF-β1 as indicated. Subsequent procedures were conducted as specified in A and B.

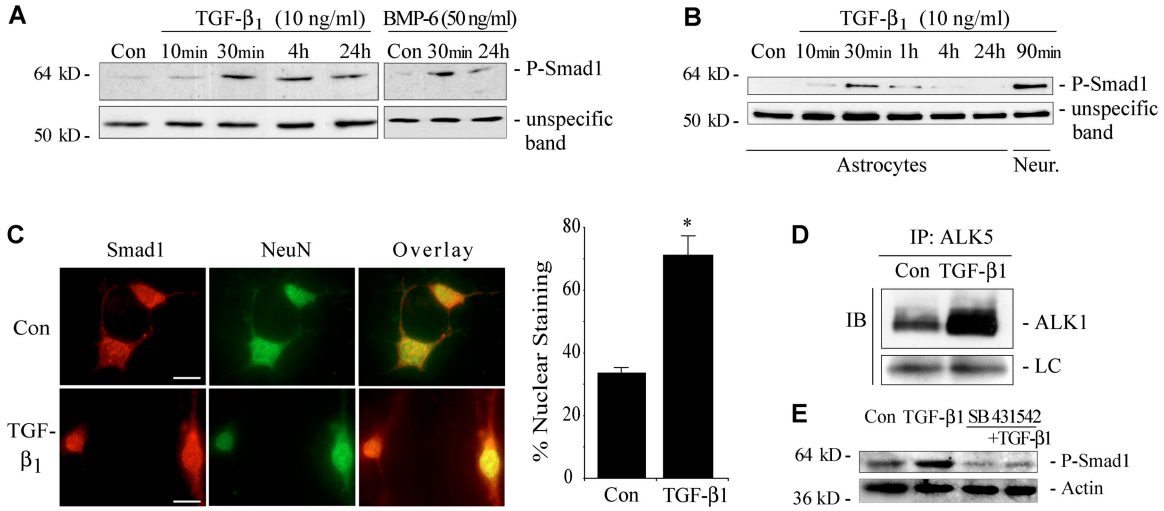

Figure 4. **TGF-β1 induces ALK1 hetero-oligomerization with ALK5 and subsequent phosphorylation of Smad1.** DIV14 hippocampal neurons (A) or secondary astrocytes (B) were incubated with the indicated cytokines, lysed, and subjected to SDS-PAGE and blotting (as described in Fig. 3 A). Membranes were incubated with P-Smad1 antibody. An unspecific band served as internal loading control as previously described (Goumans et al., 2002; compare B to Fig. 3 E, stripped membrane). The experiment was performed in triplicate with comparable results (A). (C) Immunofluorescence detection of Smad1 translocation to the nucleus and its quantitative evaluation in cultured rat hippocampal neurons. Neurons were treated with 10 ng/ml TGF-β1 for 3 h. After fixation and permeabilization, localization of Smad1 protein was determined by anti-Smad1 antibody (red). Neuronal nuclei were stained using NeuN antibody (green). Note the increase in Smad1 nuclear patches occurring in TGF-β1–treated cells, resembling neurons treated with BMP-4 (Angley et al., 2003). Bars, 10 μm. Quantification of nuclear translocation was determined without knowledge of the respective treatments. A total of 350–450 cells were counted in three separate experiments. Data are means ± SEM; *, $P < 0.05$. (D) Coimmunoprecipitation analysis of TGF-β1 induced ALK5/ALK1 receptor complexes. Cortical neurons (DIV14) were treated with10 ng/ml TGF-β1 for 15 min. After immunoprecipitation with ALK5 antibody, SDS-PAGE and Western blotting were conducted as described in Fig. 3 A. Membranes were incubated with the anti-ALK1 antibody. Immunodetection of light-chain (LC) by anti–rabbit antibody served as control. (E) Hippocampal neurons were incubated with 10 ng/ml TGF-β1 for 1 h. 30 min of preincubation with 5 μM SB-431542 were performed as indicated.

(Fig. 3 B). TGF-β1 treatment also induced significant Smad2 phosphorylation in cultured secondary astrocytes (Fig. 3 E). To examine the downstream activation of the Smad2/3-evoked transcriptional response in neurons, we used a Smad3-responsive $(CAGA)_{12}$-luciferase reporter gene assay (Dennler et al., 1998). Transient transfection experiments revealed that TGF-β1 significantly induced the activity of the $(CAGA)_{12}$-luciferase reporter gene (Fig. 3 C), whereas BMP-6 did not affect reporter gene expression. Constitutively active forms of ALK5 and ALK1 receptors have been shown to transduce signaling in a ligand-independent manner (Goumans et al., 2002). We co-transfected hippocampal neurons with plasmids encoding caALK5 and caALK1, and examined their influence on $(CAGA)_{12}$-luciferase reporter activity. Transient transfection of caALK5, but not caALK1, was able to mimic the effect of TGF-β1 on $(CAGA)_{12}$-luciferase reporter activity (Fig. 3 D).

The alternative receptor ALK1 has been reported to transduce TGF-β signaling in primary endothelial cells via a Smad1-dependent pathway (Chen and Massague, 1999; Goumans et al., 2002). Interestingly, phosphorylation of Smad1 protein occurred rapidly upon exposure of hippocampal neurons to TGF-β1 (Fig. 4 A). In contrast to Smad2 phosphorylation (Fig. 3 A), this activation exhibited a stable plateau for up to 24 h (Fig. 4 A). A similar response was observed in neurons treated with BMP-6 (50 ng/ml), which served as a positive control. In agreement with the low expression of ALK1 (Fig. 1, A and B), cultured astrocytes exhibited only transient and modest levels of phosphorylated Smad1 in comparison to neurons (Fig. 4 B). We additionally confirmed Smad1 signaling by analysis of Smad1 nuclear translocation after TGF-β1 (10 ng/ml) treatment. After a 3-h incubation period, a statistically significant increase in nuclear Smad1 immunostaining became apparent (Fig. 4 C).

Next, we sought to further confirm the role of ALK1 in neuronal TGF-β signaling. Recent evidence suggests that upon ligand binding, ALK1 associates with ALK5 and TβRII to form a kinase-active heteromeric complex, thereby requiring a functional ALK5 receptor for signal transduction (Goumans et al., 2003). Brief stimulation with TGF-β1 indeed led to increased coimmunoprecipitation of ALK1 with ALK5 in cultured neurons (Fig. 4 D). The synthetic inhibitor SB-431542 has previously been shown to block ALK5- and ALK1-dependent Smad phosphorylation (Inman et al., 2002; Goumans et al., 2003). Consequently, we evaluated ALK1-directed Smad1 phosphorylation by using SB-431542. Preincubation with the inhibitor repressed TGF-β1–induced phosphorylation of Smad1 (Fig. 4 E).

TGF-β1 treatment induces neuronal IκB-α phosphorylation and increases DNA binding of the p65 NF-κB subunit

We have previously shown that treatment of hippocampal neurons with TGF-β1 increased the expression of the NF-κB target genes Bcl-2 and Bcl-xL (Prehn et al., 1994, 1996). Therefore, we were interested to determine if TGF-β1 can induce the activation of the NF-κB pathway in neurons. Phosphorylation

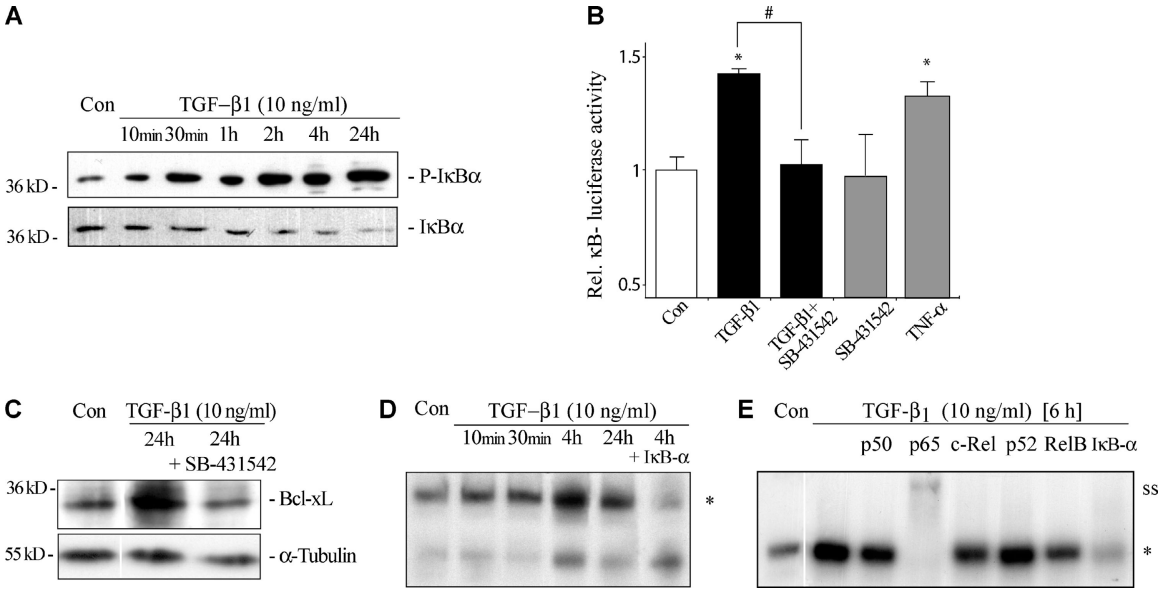

Figure 5. **TGF-β1 activates NF-κB in hippocampal neurons.** (A) DIV14 cultured rat hippocampal neurons were treated with 10 ng/ml TGF-β1 as indicated. Whole cell lysates were subjected to SDS-PAGE and Western blotting. Serine-32 phosphorylated IκB-α was immunodetected by incubation of membranes with the polyclonal P-IκB-α antibody (top). For evaluation of overall IκB-α levels, membranes were stripped and reprobed with a rabbit polyclonal IκB-α antibody (bottom). (B) Dual-luciferase NF-κB reporter gene assay. After transfection of NF-κB-luc and RL-TK-luc, cells were allowed to recover for 24 h. 30 min of preincubation with 5 μM SB-431542 preceded 24 h of exposure to TGF-β1. 10 ng/ml TNF-α treatment served as positive control. Data are means ± SEM of n = 8–16 treatments in three independent, normalized, and pooled experiments. *, #, $P < 0.05$ compared with control or TGF-β1 treatment, respectively. (C) Western blot analysis for Bcl-xL protein content after 24 h of treatment with TGF-β1 in primary rat hippocampal neurons. Cells were preincubated for 30 min with 5 μM SB-431542 as indicated. After stripping, α-tubulin served as loading control. (D) Time course of enhanced κB-DNA binding after TGF-β1 treatment as revealed by gelshift experiments. Lysates were incubated with γ^{32}P-ATP–labeled κB-consensus oligonucleotide (for comparative reasons, identical lysates as for the experiment depicted in Fig. 4 A were used). Experiments in triplicate revealed comparable results. Addition of IκB-α protein served as control for κB-binding specificity. The asterisk marks the NF-κB dimer-specific band. (E) Supershift analysis of TGF-β1–induced NF-κB complexes. After stimulation of hippocampal neurons with TGF-β1, equal amounts of lysates were analyzed by incubation with antibodies to the respective NF-κB subunits before incubation with labeled NF-κB oligonucleotide as denoted. Supershifted bands (ss) where visualized using autoradiography. A duplicate experiment yielded comparable results. (C and E) White lines indicate that intervening lanes have been spliced out.

of serine 32 and 36 of the inhibitory IκB-α and its subsequent degradation have been recognized as one of the key steps in NF-κB activation (Ghosh and Karin, 2002). TGF-β1 induced a rapid and persistent accumulation of serine32-phosphorylated IκB-α in hippocampal neurons (Fig. 5 A, top), which correlated with a slow decline in IκB-α levels (Fig. 5 A, bottom). Next, we examined the effect of TGF-β1 on NF-κB–dependent gene expression using a κB-dependent luciferase reporter assay. It is important to note in this context that neurons exhibit constitutively high levels of baseline NF-κB activity (Kaltschmidt et al., 1994). Of note, stimulation with TGF-β1 for 24 h further increased κB-dependent reporter gene expression (Fig. 5 B). Interestingly, preincubation with SB-431542 suppressed TGF-β1–stimulated NF-κB activity toward the baseline level. Exposure to TNF-α (Fig. 5 B) and repression of NF-κB liberation by overexpression of IκB-α protein (unpublished data) served as positive and negative controls, respectively. Treatment of hippocampal neurons with TGF-β1 also resulted in a marked increase in Bcl-xL protein levels, and this effect could be blocked by preincubation with SB-431542 (5 μM; Fig. 5 C). Subsequently, we analyzed the time course of NF-κB induction in gelshift experiments. TGF-β1 treatment led to a substantial increase in κB-DNA binding after 4 and 24 h post-treatment (Fig. 5 D). Increasing evidence suggests that different subunit compositions of activated NF-κB complexes regulate specific subsets of NF-κB target genes in a time- and stimulus-dependent fashion (Hoffmann et al., 2003; Saccani et al., 2003). To identify the NF-κB subunit composition induced by TGF-β1 in hippocampal neurons, we conducted supershift experiments. Antibodies directed against p65 were able to supershift the TGF-β1–induced NF-κB–DNA complex, whereas antibodies to other subunits (p50, p52, c-Rel, and RelB) caused no discernible shift (Fig. 5 E).

The constitutively active mutant of ALK1 mimics TGF-β-dependent Smad1 phosphorylation, NF-κB activation, and neuroprotection

To reveal whether ALK1 or ALK5 mediate NF-κB activation in hippocampal neurons, we cotransfected hippocampal neurons with caALK1 and caALK5 in combination with p65-EGFP. The fusion protein p65-EGFP has been shown to be a valuable tool in visualizing p65/RelA nuclear translocation (Meffert et al., 2003), which is a prerequisite for NF-κB–dependent transcription (Ghosh and Karin, 2002). To confirm p65-EGFP localization in hippocampal neurons, costaining with the neuronal marker βIII-tubulin was used. Interestingly, caALK1 led to a markedly increased nuclear accumulation of p65-EGFP in the hippocampal neurons 24 h after

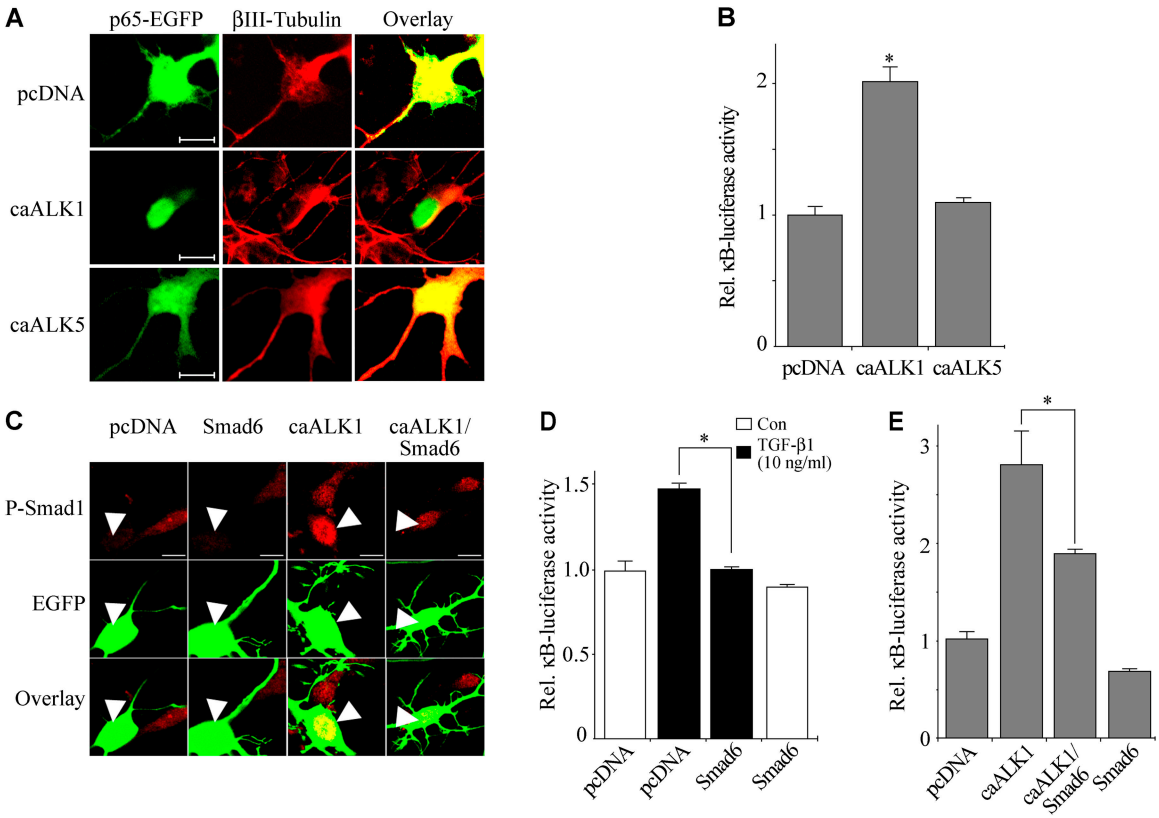

Figure 6. **ALK1 mediates TGF-β–induced NF-κB activity, whereas overexpression of Smad6 abrogates this effect.** (A) Primary rat hippocampal neurons were transfected with p65-EGFP and cotransfected with the constitutively active ALK encoding plasmids. 24 h after transfection, cells were fixed and stained with antiserum to neuronal marker βIII-tubulin (red). Localization of p65-EGFP (green) was evaluated using confocal microscopy as exemplified in the figure. In preliminary experiments, the amount of p65-EGFP was titrated down to a level still showing a homogeneous somatic distribution in a majority of neurons. Functionality of p65-EGFP was proven by its ability to increase NF-κB-luciferase reporter gene (unpublished data). Bars, 10 μm. (B) Dual-luciferase reporter gene assay for enhanced NF-κB activity after cotransfection of constitutively active TGF-β type I receptors. Hippocampal neurons were transfected on DIV6 with NF-κB-luciferase and RL-TK-luciferase reporter gene constructs, as well as cotransfected with either caALK1, caALK5, or pcDNA vector. 48 h after transfection, cells were lysed and dual-luciferase assay was performed. (C) Confocal immunofluorescence of increased nuclear accumulation of phosphorylated Smad1 in caALK1-transfected neurons. Hippocampal neurons were transiently cotransfected with pEGFP-N1 (green) and either control pcDNA, caALK1, or Smad6 expression vectors as indicated. 24 h after transfection, cells were fixed and immunostained with anti-P-Smad1 antibody (red). Arrowheads indicate cotransfected cells with increased versus decreased nuclear P-Smad1 staining. Bars, 10 μm. (D) Hippocampal neurons were transfected with the NF-κB-luciferase reporter plasmids and cotransfected with control or Smad6 vector after which they were allowed to recover from transfection for 24 h. Subsequent treatment with TGF-β1 was performed for a period of 24 h. (E) NF-κB-luciferase reporter gene transfected neurons were cotransfected with the indicated plasmids. 48 h after transfection, a dual-luciferase assay has been performed. Experiments performed in duplicate yielded similar results. The data represent NF-κB activity in relation to control ± SEM from $n = 6$ wells. *, $P < 0.05$ as compared with control or bracketed treatment.

transfection. In contrast, caALK5 alone did not induce significant p65-EGFP translocation (Fig. 6 A). We further determined whether or not ectopic expression of caALK1 was sufficient to potently stimulate NF-κB reporter gene activity. Expression of caALK1 induced a potent increase in κB reporter gene transcription, whereas caALK5 could not elicit a substantial increase compared with control transfections (Fig. 6 B).

The inhibitory Smad6 protein specifically interferes with Smad1/5-mediated signal transduction (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200407027/DC1; Hata et al., 1998; Zhao et al., 2000). Transfection of caALK1 substantially elevated nuclear immunofluorescence of phosphorylated Smad1 in the hippocampal neurons. In contrast, cotransfection of caALK1 with Smad6 abolished the elevated nuclear immunofluorescence of phosphorylated Smad1 (Fig. 6 C).

To test whether or not ALK1 activation is required for TGF-β1–induced NF-κB activation, we cotransfected hippocampal neurons with the inhibitory Smad6 protein and performed NF-κB reporter gene assays in vehicle and TGF-β1–treated hippocampal neurons. Interestingly, the induction of NF-κB activity by TGF-β1 could efficiently be blocked by Smad6 cotransfection (Fig. 6 D). In line with these observations, cotransfection of Smad6 protein with caALK1 also led to significant abrogation of caALK1-evoked NF-κB activation (Fig. 6 E), whereas transfection of Smad6 alone exhibited no significant change in κB-dependent reporter gene expression (Fig. 6, D and E).

Finally, we tested if caALK1 is able to also mimic TGF-β1–mediated neuroprotection. Preincubation with TGF-β1 has previously been shown to rescue neurons from excitotoxic, ischemic, and oxidative stress–induced cell death (Henrich-Noack et al., 1996). Cortical neurons were trans-

fected with caALK1 and caALK5 expression vectors and exposed to the nitric oxide (NO)–generating agent S-nitrosocysteine (SNOC; 30 μM; Henrich-Noack et al., 1996). NO is an important mediator of both excitotoxic and ischemic neuronal injury (Dawson et al., 1991; Ayata et al., 1997). Indeed, overexpression of caALK1 in neurons conferred a substantially enhanced resistance against SNOC-induced cell death, whereas neurons transfected with caALK5 were not protected (Fig. 7 A).

Discussion

In the present work, we demonstrate constitutive expression and stress-induced up-regulation of the novel TGF-β type I receptor ALK1 in neurons. Furthermore, we provide evidence that TGF-β1 and ALK1 activate the transcription factor NF-κB in a manner depending on an intact Smad1/5 pathway. Constitutive and inducible NF-κB activity has been shown to contribute to neuronal survival and to protect the nervous system from various stress conditions (Kaltschmidt et al., 1994; Mattson et al., 2000). Our data, together with previous work on TGF-β1–mediated neuroprotection, suggest that the concomitant up-regulation of TGF-β1 and ALK1 expression during brain injury may be an essential part of an endogenous response aimed to limit the consequences of metabolic, toxic, or traumatic injury to the nervous system.

Although microglia and macrophages appear to be a major source of TGF-β1 during brain injury (Lindholm et al., 1992), the corresponding ALK1 receptor is expressed at high levels particularly in neurons (Figs. 1 and 2). This finding suggests the existence of a framework for a direct crosstalk between activated microglia and neurons during brain injury. Early studies on TGF-β signaling described ALK1 as an alternative type I receptor for TGF-β (Franzen et al., 1993). A physiological role of TGF-β1–induced ALK1 activation has so far only been described in vascular endothelial cells (Oh et al., 2000). TGF-β1 signaling in vascular endothelial cells involves the canonical ALK5-mediated Smad2/3 pathway, as well as ALK1-directed phosphorylation of Smad1/5 (Goumans et al., 2002). This pathway was initially described as a BMP-restricted pathway, transduced through distinct BMP receptor subsets (Shi and Massague, 2003). In endothelial cells, the intricate balance between ALK1 and TβRI/ALK5 signaling orchestrates epithelial to mesenchymal transition in TGF-β regulation of angiogenesis (Oh et al., 2000; Goumans et al., 2002). Our work demonstrates a simultaneous TGF-β1 activation of the ALK5/Smad2/3 and the ALK1/Smad1/5 pathway in the hippocampal neurons. TGF-β1 induced an increase in ALK5-mediated phosphorylation of Smad2, as well as an increase in Smad3-specific $(CAGA)_{12}$-luciferase reporter gene activity. Interestingly, phosphorylation of Smad2 in the hippocampal neurons was only transient, and the expression level of ALK5 was moderate compared with that observed in astrocytes or fibroblasts. Moreover, in contrast to astrocytes, ALK1 expression and ALK1-dependent Smad1 phosphorylation by TGF-β1 was much more pronounced and persistent in neurons, suggesting that ALK1 may be of greater importance

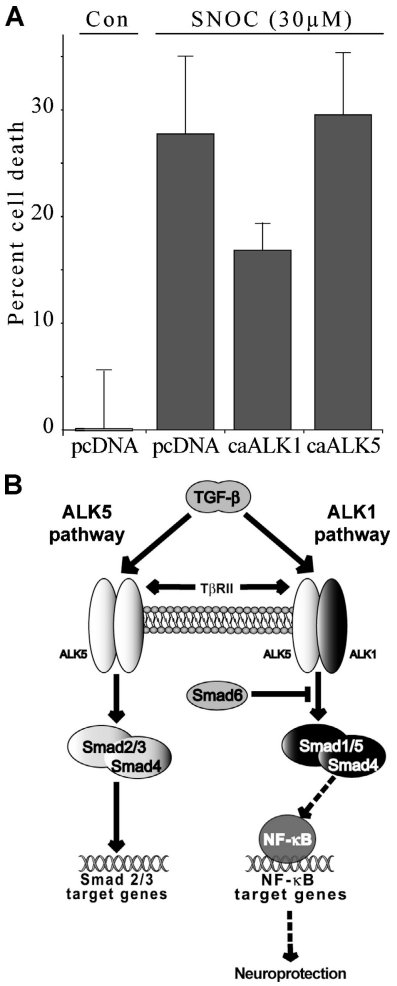

Figure 7. **ALK1-initiated signaling confers neuroprotection.** (A) Expression of the constitutively active ALK1 receptor results in increased resistance to oxidative stress. Cultured primary cortical neurons were cotransfected with caALK1, caALK5, or control vector together with EGFP. After transfer to antioxidant-depleted media, cultures were treated with 30 μM of the NO-donator SNOC. 24 h after treatment, cultures were incubated with PI for evaluation of cell death. 910–1226 EGFP-positive neurons were counted and depicted as means ± SEM of $n = 3$–6 treatments in two normalized and pooled experiments. (B) Proposed schematic model for TGF-β–induced downstream signaling in hippocampal neurons. TGF-β binds to TβRII, which recruits and activates the type I receptors. ALK1 and ALK5 phosphorylate their downstream targets Smad1/5 or Smad2/3, respectively. Moreover, Smad6 specifically inhibits the ALK1-induced pathway. Smad1/5-involving signaling is able to evoke NF-κB activation, whereas Smad2/3 induction results in activation of the classical TGF-β target genes.

in neuronal TGF-β1 signaling. Hence, we propose a model in which the relative balance of ALK1 and ALK5 expression may determine the composition of activated receptor complexes in a competitive manner, thus leading to transduction of TGF-β–induced signals into a predominant downstream pathway (Fig. 7 B).

Activation of the Smad1 pathway by TGF-β1 in neurons was associated with activation of NF-κB. Interestingly, a non-phosphorylatable mutant of Smad1 has been shown to prevent Smad1-dependent proteasomal degradation of the Smad nuclear interacting protein 1, which negatively regulates the NF-κB pathway by sequestration of the p65 coactivator CBP/p300

(Wang, 2003). Of note, enhanced NF-κB activity in neurons after TGF-β1 stimulation was associated with the activation of the p65 NF-κB subunit. The formation of p65/p65 homodimers has been implicated in NF-κB signaling (Ganchi et al., 1993). Several studies have substantiated the concept that distinct NF-κB subunit compositions coordinate the activation of distinct subsets of NF-κB target genes (Hoffmann et al., 2003; Saccani et al., 2003). We could demonstrate a potent TGF-β1–dependent up-regulation of the antiapoptotic protein Bcl-xL. Because *Bcl-x* has previously been identified as a p65 target gene in neurons (Bhakar et al., 2002), induction of Bcl-xL via NF-κB might constitute an integrative mechanism by which neuroprotective cytokines such as TGF-β1 and NGF (Bui et al., 2001) promote survival of neurons. Overexpression of the inhibitory Smad6 protein was able to abrogate the effects of TGF-β1 and Smad6 on NF-κB activation. Although Smad7 has been implicated in blockade of Smad2/3 as well as Smad1/5-induced signaling (Shi and Massague, 2003), Smad6 has been shown to specifically inhibit Smad1/5-mediated cellular responses (Fig. S1; Hata et al., 1998; Zhao et al., 2000). Interestingly, NF-κB has been shown to up-regulate Smad7 expression (Bitzer et al., 2000), which may actually lead to a suppression of the canonical, ALK5-mediated TGF-β1 signaling pathway in neurons. In addition to ALK1-mediated survival signaling in neurons, the ALK5/Smad2/3 pathway may promote specific astroglial responses such as up-regulation of plasminogen activator inhibitor type 1 (Buisson et al., 1998), and thus might contribute to TGF-β–induced protective tissue responses during brain injury.

TGF-β1 and TGF-β3 have been identified as ligands to the ALK1 receptor, whereas TGF-β2 shows very little affinity to this receptor (Lux et al., 1999). It is interesting to note in this context that TGF-β1 and TGF-β3 have suggested to be more potent in protecting neurons from hypoxic and excitotoxic injury than TGF-β2 (Prehn et al., 1993b), suggesting nonredundant functions of the TGF-β family members. Similar to effects of TGF-βs in hepatocytes and immune cells, TGF-βs have also been shown to be capable of inducing or potentiating cell death in neurons (Prehn and Krieglstein, 1994; de Luca et al., 1996). Likewise, immunoneutralization of endogenous TGF-βs has been shown to reduce ontogenetic cell death in a subset of neuronal populations (Krieglstein et al., 2000). Possibly, the conflicting reports on protective versus death-promoting abilities of the TGF-βs can directly be explained by the balance between ALK1 and ALK5 receptor-related signaling cascades, differences in ALK1 and ALK 5 receptor expression, as well as potentially by nonredundant biological activities of the three isoforms. In support of the latter, TGF-β2 was recently identified as a potential inhibitor of neuronal survival signaling in an unbiased genomic screen approach (Kaltschmidt and Kaltschmidt, 2001). TGF-βs may hence enable the nervous system to fine-tune both developmental processes and brain responses to injury. From a teleological point of view, TGF-βs may join other neurotrophic factors such as NGF in their intricate ability to influence cell survival and cell death decisions by activation of different subsets of receptors (Barrett and Bartlett, 1994; Carter et al., 1996).

Materials and methods

Materials

Human recombinant TGF-β1 and BMP-6 were obtained from R&D Systems and TNF-α came from Biosource International. SB-431542 and tetrodotoxin were obtained from Tocris Cookson Ltd. All other chemicals were obtained from Sigma-Aldrich or Applichem. Cell culture media and supplements came from Invitrogen unless otherwise indicated.

Cell culture

Rat embryonal fibroblast (REF52), hepatoblastoma (HepG2), as well as rat pheochromocytoma (PC12) cells, and astrocytes were grown in DME supplemented with 10% FCS and the antibiotic mixture of 100 U/ml penicillin and 100 μg/ml streptomycin. Rat hippocampal neurons were prepared from neonatal (P0–P1) Wistar rats and maintained as described previously (Kögel et al., 2004). Cortical neurons were prepared as previously outlined (Dawson et al., 1991) and maintained in Neurobasal medium with 2% B-27 supplement, 2 mM L-glutamine, and the antibiotic mixture. Neuronal cultures received cytosine-β-D-arabinofuranoside (CAF, 1 μM; Sigma-Aldrich) beginning on days in vitro (DIV) 2 to suppress glial proliferation. Secondary cultures of astrocytes from the cortex of neonatal Wistar rats (P1) were prepared as described previously (Kögel et al., 2004). After 10 d in culture, cells were passaged and kept in CAF-free hippocampal medium for treatment with TGF-β1. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. Animal care followed official governmental guide lines.

RT-PCR

Extraction of total cellular RNA, reverse-transcription, and PCR was performed as previously described (Kögel et al., 2004). Primer sequences and amplification procedures are available in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200407027/DC1).

Immunofluorescence analyses and immunocytochemistry

Cells were fixed with 4% PFA and permeabilized by PBS/0.1% Triton X-100. For ALK1 immunofluorescence analyses, the prior permeabilization step was omitted. Nonspecific antibody binding was blocked by PBS/5% horse serum/0.3% Triton X-100 for 30 min at RT in all cases. Antibodies were diluted in blocking buffer. Incubation times followed general procedures. ALK1 was detected using a goat polyclonal anti-ALK1 antibody (D-20; Santa Cruz Biotechnology, Inc.), and blocking peptide (19546 P; Santa Cruz Biotechnology, Inc.) was used at 20-fold excess by weight. After permeabilization, mouse mAbs for neuronal marker proteins βIII-tubulin (Promega) or anti-NeuN (Chemicon), the glial fibrillary acidic protein anti-GFAP (Promega), as well as a rabbit polyclonal anti-Smad1 antibody (Upstate Biotechnology), and a rabbit polyclonal antiphosphorylated Smad1 antibody (Upstate Biotechnology) were used to detect the respective proteins. Secondary antibodies used were either biotin-conjugated anti–goat, anti–rabbit, or anti–mouse IgG (Vector Laboratories). Subsequent incubation was conducted with streptavidin-conjugated Oregon green or Texas red (Molecular Probes). Coronal brain sections (provided by C. Schultz, University Clinics Frankfurt, Frankfurt, Germany) were obtained from female adult rats perfused transcardially with cold 4% PFA under anesthesia. DAB histochemistry followed general procedures. Sections or cells were mounted with Permafluor (Immunotech). Light or immunofluorescence microscopy was performed at RT using an inverted microscope (Eclipse TE2000S; Nikon) and a 60× oil immersion objective (NA 1.40; Nikon). Texas red or Oregon green/EGFP-fluorescence was observed using the following optics: excitation, 540–580 nm; dichroic mirror, 595 nm; emission, 600–660 nm; or excitation, 465–495; dichroic mirror, 505; emission, 515–555 nm, respectively. Confocal images and stacks were acquired on a microscope (model LSM 510; Carl Zeiss MicroImaging, Inc.) with a krypton/argon and helium/neon laser and a 63× oil immersion objective (NA 1.40; Carl Zeiss MicroImaging, Inc.). For p65-EGFP or Oregon green, the excitation/emission filter was 488/505–530 nm. For Texas red and propidium iodide (PI) staining, excitation/emission was 543/560–615 nm.

Immunoprecipitation analysis, SDS-PAGE, and Western blotting

Cell lysis for Western blotting was performed using TOTEX (Kögel et al., 2004) or RIPA lysis buffer, as indicated. For immunoprecipitation analysis, cells were solubilized with PBS containing 1% (vol/vol) Triton X-100, 5 g/liter sodium deoxycholate, and 1 g/liter SDS. Immunoprecipitation was performed using rabbit polyclonal anti-TβRI–conjugated agarose beads (V-22; Santa Cruz Biotechnology, Inc.) according to the manufacturer's instructions. All lysis buffers contained protease inhibitors (Protease

inhibitor cocktail; Sigma-Aldrich). SDS-PAGE, Western blotting, and stripping procedures were conducted as described previously (Bui et al., 2001). Immunodetections were performed with a goat polyclonal anti-ALK1 antibody (D-20; Santa Cruz Biotechnology, Inc.), a monoclonal anti-rat Bcl-x antibody (B22620; Transduction Laboratories), and a mouse monoclonal anti–α-tubulin antibody (Sigma-Aldrich), as well as rabbit polyclonal antibodies against serine 465/467-phosphorylated Smad2 (P-Smad2; Upstate Biotechnology), serine 463/465-phosphorylated Smad1 (P-Smad1; Upstate Biotechnology), serine 32-phosphorylated IκB-α protein, and total IκB-α (Cell Signaling Technology) according to the manufacturer's instructions.

Electrophoretic mobility shift assay (Gelshift)
Gelshift analysis was performed as described previously (Kögel et al., 2004). For supershift experiments, the respective antibodies corresponding to NF-κB subunits (Santa Cruz Biotechnology, Inc.) were added to the mixture before the γ^{32}P-ATP–labeled NF-κB consensus oligonucleotide (Promega). Samples were separated on 4% nondenaturing gels and analyzed by autoradiography.

Transient transfection experiments and analysis of reporter gene activity
For reporter gene studies, hippocampal neurons were transfected in 24-well plates (Becton Dickinson) using the Lipofectamine 2000 (Invitrogen) transfection reagent following the manufacturer's protocol. Transfection efficiency, revealed by cotransfection experiments with pEGFP-N1 (CLONTECH Laboratories, Inc.) ranged between 10–30% in control experiments. Transfection mixes contained 0.3 μg/well of pcDNA 3.1 (Invitrogen), pcDNA-ALK1 (Q201D)/caALK1 (provided by C.-H. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden), pcDNA-ALK5 (T204D)/caALK5, or pcDNA-Smad6/Smad6 (provided by M. Kawabata, The Cancer Institute, Tokyo, Japan) expression vector. Cells were cotransfected with 0.3 μg/well of a plasmid containing six tandem repeats of the κB enhancer element upstream of the coding sequence of the Firefly-luciferase (NF-κB-luc) or a plasmid encoding TGF-β responsive $(CAGA)_{12}$-luciferase (Dennler et al., 1998; provided by Glaxo-Wellcome, Les Ulis, France) along with 0.05 μg of a plasmid coding for the Renilla-luciferase (phRL-TK-luc; Promega) as transfection control for normalization in dual-luciferase assays. Dual-Luficerase reporter assay was performed following the manufacturer's protocol (Promega). For immunofluorescence analysis, rat hippocampal neurons were transfected with a plasmid encoding p65 fused to the fluorescent EGFP (p65-EGFP; provided by M. Rowe and E. Floettmann, University of Wales College of Medicine, Cardiff, UK) or pEGFP-N1 (CLONTECH Laboratories, Inc.), respectively, together with the TβRI mutants.

Induction of neuronal injury and evaluation of cell death
Induction of excitotoxic neuronal injury was performed as described previously (Kögel et al., 2004). For the induction of NO-mediated toxicity, cultured cortical neurons were transfected with the respective TβRI receptor mutants, together with pEGFP-N1 as described for transient transfection experiments. After transfection, every 12 h, half of the medium was exchanged with the conditioned neurobasal medium containing antioxidant-free B27 (Invitrogen) to slowly deplete antioxidants. 36 h after transfection, all media were replaced by the conditioned antioxidant-free media and the freshly prepared NO-generating agent SNOC (Lei et al., 1992) was added to the culture medium at 30 μM as previously described (Henrich-Noack et al., 1996). Aged SNOC, left at RT for 2 d, served as control. 24 h after toxicity induction, cells were stained with 2 μg/ml PI and immediately assayed for cell death. PI/EGFP-positive neurons were counted as dead versus total number of EGFP-positive neurons.

Induction of temporal cerebral ischemia by MCAO
Experiments were performed on adult male Sprague-Dawley rats (R. Janvier Breeding Center, Le Genest-St Isle, France) under anesthesia during surgery according to the appropriate European Directives. Temporary focal cerebral ischemia was induced by occlusion of the right middle cerebral artery by use of the intraluminal filament technique (Longa et al., 1989). A nylon thread was inserted to the origin of the middle cerebral artery and removed 60 min later to allow reperfusion. For sham-operated animals, the nylon thread was immediately removed. Rat brain homogenates enriched for the lesioned areas containing cortex and striatum were analyzed by Western blotting.

Statistics
Data are presented as means ± SEM. For statistical comparison, one-way ANOVA followed by Tukey's test was used. Mann-Whitney U-test was used for evaluation of nonparametric data. P values <0.05 were considered to be statistically significant.

Online supplementary material
Table S1shows primer sequences and amplification procedures as conducted for RT-PCR experiments. Additional reporter gene assays have been conducted to confirm specific blocking of the ALK1-induced NF-κB pathway by Smad6 (Fig. S1). Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200407027/DC1.

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