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Smad3 deficiency increases cortical and hippocampal neuronal loss following traumatic brain injury

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ABSTRACT

Transforming growth factor- β (TGF- β) signaling is involved in pathological processes following brain injury. TGF- β signaling through Smad3 contributes significantly to the immune response and glial scar formation after brain injury. However, TGF- β is also neuroprotective, suggesting that Smad3 signaling may also be involved in neuroprotection after injury. We found expression of the TGF- β type II receptor (T β RII) and Smad3 protein to be strongly and rapidly induced in neurons in the ipsilateral cortex and CA1 region of the hippocampus after stab wound injury. In contrast, astrocytic expression of T β RII and Smad3 was induced more slowly. Comparison of the response of wild-type and Smad3 null mice to cortical stab wound injury showed a more pronounced loss of neuronal viability in Smad3 null mice. Neuronal density was more strongly reduced in Smad3 null mice than in wild-type mice at 1 and 3 days post lesion in both the ipsilateral cortex and hippocampal CA1 region. Fluoro-Jade B, TUNEL staining, and cleaved caspase-3 staining also demonstrated increased neuronal degeneration at early time points after injury in the ipsilateral hemisphere in Smad3 null mice. Taken together, our results suggest that TGF- β cytokine family signaling through Smad3 protects neurons in the damaged cortex and hippocampus at early time points after injury.

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Introduction

Transforming growth factor- β (TGF- β) is widely recognized as a critical regulator of key events in development, disease, and repair (Dunker and Kriegstein, 2000; Massague, 1998). TGF- β regulates inflammatory responses after injury in the peripheral and central nervous system (CNS) (Wyss-Coray et al., 1997), and functions as a survival factor for embryonic motoneurons, dopaminergic and neonatal sensory neurons in vitro (Chalazonitis et al., 1992; Kriegstein et al., 1995; Martinou et al., 1990; Poulsen et al., 1994). TGF- β exerts neurotrophic effects on damaged neurons (Prehn et al., 1993), rescues sympathetic neurons from death after destruction of the target cells (Blottner et al., 1996), and protects dopaminergic neurons from *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity and cortical neurons from sodium cyanide or glutamate (Farkas et al., 2003; Kriegstein et al., 1995; Prehn et al., 1993). Further, antagonism of TGF- β receptor signaling led to increased neurodegeneration in models of either excitotoxic injury or Alzheimer's disease (Ruocco et al., 1999;

Tesseur et al., 2006). Thus, TGF- β signaling is critical for the brain injury/repair response under a variety of different conditions.

TGF- β is induced after traumatic brain injury, and may act as a neuroprotective factor in the injured CNS (Lindholm et al., 1992; Logan et al., 1999b; Stoll et al., 2004). However, after brain injury TGF- β is also responsible for deposition of extracellular matrix proteins that contribute to glial scar formation (Logan et al., 1994, 1999a) which retards neuronal regeneration (McKeon et al., 1995; Windle et al., 1992) and increases the induction of traumatic epilepsy after brain injury (Hoepfner and Morrell, 1986; Tian et al., 2005). Additionally, TGF- β may suppress the early stages of neurogenesis (Buckwalter et al., 2006; Wachs et al., 2006), which may reduce the regenerative capacity of the brain. Thus, targeting TGF- β to enhance recovery from traumatic injury becomes complex because of these multiple and sometimes conflicting functions. It is therefore critical to identify the TGF- β transduction pathways and downstream effectors through which TGF- β mediates individual effects in an effort to target specific pathways mediating detrimental effects while leaving its beneficial effects unaltered.

Active TGF- β induces the formation of receptor hetero-tetramers that contain two "type I" (T β R-I) and two "type II" (T β R-II) transmembrane serine threonine kinase receptors (Vilar et al., 2006). TGF- β isoforms elicit their specific responses through the ligand-induced formation of a heteromeric receptor complex between T β R-I and T β R-II. T β R-II

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binds TGF- β first, and then recruits T β R-I, allowing for the transphosphorylation-mediated activation of T β R-I. This ligand–receptor activation leads to phosphorylation of Smad2 and Smad3 by the type I receptor, and their release from a cytoplasmic protein (reviewed in (Derynck and Zhang, 2003; Heldin et al., 1997; Krieglstein et al., 1995)). These receptors activate Smad proteins (R-Smads) which then form heteromeric complexes with the pathway-independent Smad4 (Lagna et al., 1996). The Smad complex translocates to the nucleus where it regulates transcription of TGF- β target genes (Massague et al., 2005). Smads interact with a diverse array of transcription factors to bring about TGF- β regulated transcription (Feng and Derynck, 2005; ten Dijke and Hill, 2004). However, although Smad proteins are critical to many of the actions of TGF- β , TGF- β utilizes several different pathways for signaling within cells, including Smad-independent pathways (Farkas et al., 2003; Massague, 2003). Indeed, it has been proposed that the neuroprotective effects of TGF- β may be transduced by the alternative T β R-I, Alk1 signaling through NF- κ B (Konig et al., 2005).

Smad proteins are involved in many of the same neuroprotective or neurodegenerative processes that have been attributed to TGF- β , suggesting that TGF- β signaling through Smad2 or Smad3 is critical for many of these effects. Elevation of TGF- β levels after traumatic brain injury correlate with local and rapid activation of Smad2/3-dependent signaling in the lesioned brain (Lin et al., 2005). Activation of Smad3-dependent signaling in astrocytes protects neurons against NMDA-induced cell death (Docagne et al., 2002). Studies on Smad3 deficient mice have revealed a role for Smad3 in trophic support for nigral dopaminergic neurons (Tapia-Gonzalez et al., 2011), in addition to maintenance of neurogenesis and migration along the rostral migratory stream to the olfactory bulb (Wang and Symes, 2010). Dysregulation of TGF- β -Smad signaling by Tau hyperphosphorylation may contribute to the neurodegeneration inherent in Alzheimer's disease (Baig et al., 2009; Ueberham et al., 2006).

Our previous study showed that Smad3 deficient mice had reduced scar formation and less immune infiltration after a traumatic brain injury, suggesting a beneficial effect of Smad3 deficiency on recovery (Wang et al., 2007). However, it remains unknown if Smad3 plays a role in neuronal survival after traumatic brain injury. In the present study, we used these Smad3 deficient mice to investigate the potential neuroprotective role of Smad3 in the damaged cortex and hippocampus after cortical stab wound injury.

Materials and methods

Animals

Mice homozygous for a targeted deletion in exon 8 of the Smad3 gene (Smad3^{ex8/ex8}) and their wild-type littermates were bred from heterozygous Smad3^{ex8/+} mice on a mixed C57BL/6 $\times$ SV129 background. All experiments were performed on mice between 2 and 3 months old. Genotype was determined by PCR analysis of genomic DNA isolated from tail clippings at 3 weeks of age. Presence of the wild-type Smad3 allele was established using primer 1: 5'-cca ctt cat tgc cat atg ccc tg-3' with primer 2: 5'-ccc gaa cag ttg gat tca cac-3'. The targeted deletion was detected with primer 1 and primer 3: 5'-cca gac tgc ctt ggg aaa agc-3' (Yang et al., 1999). The protocols in this study were approved by the USUHS IACUC and were in accordance with the animal welfare guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care.

Stab injury

Cortical stab wound injury was carried out as previously described (Wang et al., 2007). Briefly, mice were anesthetized with ketamine/xylazine solution (50 mg/kg ketamine/7.5 mg/kg xylazine in 0.9% NaCl solution) and placed in a stereotaxic frame. A 1.5-mm groove was drilled in the cranium 2 mm caudal to the Bregma, 1 mm lateral to the midline. A 1.1-mm sterile sharp knife was inserted vertically into the right

cerebral hemisphere 2 mm deep to the dura surface. The knife was left in situ for 1 min. The injury site was covered with sterile bone wax; the skin incision was closed with sutures. At different times (1, 3, 7 or 14 days post lesion (dpl)) mice were deeply anesthetized then perfused with ice-cold fresh 4% paraformaldehyde solution.

Preparation of tissue

Brains were fixed in 4% paraformaldehyde for 24 h at 4 °C, cryoprotected in 10%, 20% and 30% sucrose for 24 h individually, then quick frozen on dry ice and stored at -80°C . 10 μm coronal sections were cut sequentially through the entire lesion site on a cryostat, mounted on gelatin coated glass slides and stored at -80°C until use. Six sections spaced 100 μm apart from each other through the width of the lesion were mounted on each slide to provide evenly distributed sections for each antibody stain.

Fluorescence immunohistochemistry

Sections taken from the -80°C freezer were washed with 0.1% Triton X-100 in 0.1 M phosphate buffered saline (PBST), blocked in PBST/10% normal goat serum (NGS) for 1 h, then incubated overnight at 4 °C in PBST/1% NGS containing the primary antibody. Sections were then washed in PBST three times and incubated with the corresponding secondary antibodies for 2 h at room temperature. For double labeling of Smad3/NeuN and Smad3/GFAP, the two primary antibodies were incubated simultaneously with the sections, as were the correspondent secondary antibodies after washing with PBST. Sections were rinsed once with PBST followed by a distilled water rinse before coverslipping with ProLong Gold antifade reagent (Invitrogen). The following primary antibodies were used: anti-NeuN (mouse monoclonal, 1:200, Millipore); anti-GFAP (mouse monoclonal, 1:1000; Millipore); anti-T β RII (rabbit polyclonal, 1:15,000; Santa Cruz Biotech); anti-Smad3 (rabbit polyclonal, 1:100; Upstate); and anti-pSmad3 (rabbit polyclonal, 1:1000, Cell Signaling). Secondary antisera were either anti-mouse or anti-rabbit IgG conjugated to Alexa Fluor 488 or Alexa Fluor 568 (1:1000, Invitrogen). Sections were also incubated with secondary antisera alone to control for non-specific binding of the secondary antiserum to the mouse brain sections. For costaining of Smad3/T β RII and pSmad3/T β RII, staining was performed sequentially, as both primary antibodies were raised in rabbit. Sections were blocked overnight in PBST/10% NGS before overnight incubation with the first primary antiserum in PBST/1% NGS. After washing, sections were incubated with the corresponding secondary antiserum for 2 h, before blocking again (PBST/10% NGS) overnight to avoid cross-reactivity. Incubation with the second primary antiserum was again performed overnight in PBS/1% NGS before a 2 h incubation with the second secondary. Sections were washed with PBS followed by rinsing in distilled water before coverslipping with ProLong Gold antifade reagent. Images (20 $\times$) were acquired using an Olympus BX61 fluorescence microscope attached to a Retiga EXi Aqua CCD camera (Qimaging), using iVision software (BioVision Technologies). For colocalization studies, double-stained cells were analyzed using a Zeiss confocal-laser scanning microscope (LSM 510) equipped with argon and He/Ne laser emitting at 488 nm and 568 nm.

Assessment of cell damage

Fluoro-Jade B histofluorescence was performed as described (Schmued et al., 1997). Briefly, brain sections were removed from the -80°C and left at room temperature for 20 min. Sections were sequentially immersed in 100% ethyl alcohol (3 min), 70% alcohol (1 min) then water (1 min). They were then immersed in 0.06% potassium permanganate for 15 min while shaking gently. After a distilled water rinse they were transferred to the 0.001% Fluoro-Jade B staining solution for 30 min. Sections were rinsed three times with

distilled water, dried and the slides immersed in xylene and coverslipped with DPX mounting media (Aldrich Chem. Co., Milwaukee, WI).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. The TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis. Sections were first labeled with anti-NeuN to determine the amount of neuronal apoptosis. Brain sections were washed with 0.1% Triton X-100 in 0.1 M phosphate buffered saline (PBST), blocked in PBST/10% normal goat serum (NGS) for 1 h, then incubated overnight at 4 °C in PBST/1% NGS containing anti-NeuN. Sections were then washed in PBST three times and incubated with anti-mouse secondary antibody for 2 h at room temperature. Sections were permeabilized with PBST and subsequently washed with PBS at room temperature. Then, the area around the brain section was dried and added to the TUNEL reaction mixture (fluorescence In Situ Cell Death Detection Kit, Roche, IL, USA) consisting of 50 μ l enzyme solution (vial 1) and 450 μ l label solution (vial 2). Brain sections were incubated at 37 °C for 60 min in the dark. Sections were then washed with PBS followed by a distilled water rinse before coverslipping with ProLong Gold antifade reagent.

Quantification and statistical analysis

Immunohistochemistry or Fluoro-Jade B staining was performed on sections spaced at 100 μ m from each other throughout the width of the lesion. The total number of Fluoro-Jade B cells in the contralateral and ipsilateral cortex and hippocampus were counted in each of 5–6 sections for each animal (100 μ m interval), for each of 6 animals and averaged. NeuN, Smad3, and T β RII immunoreactive cells were counted in the area adjacent to the lesion cavity within a 0.01 mm² window (100 μ m $\times$ 100 μ m) in the cortex and a 0.04 mm² window (200 μ m $\times$ 200 μ m) in the hippocampal CA1, for each of 5–6 sections (100 μ m interval) per animal and subsequently averaged. The intensity of immunohistochemical signal in the Smad3+ or NeuN+ cells was measured with metamorph software (Molecular Devices, Sunnydale, CA). Individual cellular Smad3 and NeuN immunoreactive intensity was determined through outlining the region around each cell, thresholding the image and subtracting the background from the intensity reading (Mokin and Keifer, 2006). All exposure parameters remained identical during image capture. Data are presented in gray scale values. For statistical analysis, the cell number or the gray level value was averaged from all the sections measured for each mouse. Differences between wild-type (+/+) and Smad3 null (–/–) mice at different time points were determined by two-way ANOVA with Bonferroni or Sidak corrections for multiple comparisons (Prism 6 software, Graphpad). All other comparisons were calculated by one-way ANOVA using the Bonferroni multiple comparison test.

Results

T β RII expression is rapidly induced in neurons in the cortex and hippocampus after stab wound injury

T β RII is the specific type II ligand binding receptor subunit for TGF- β and is absolutely required for TGF- β signal transduction whereas there are at least 2 type I receptors that can complex with T β RII to transduce TGF- β signals (de Caestecker, 2004). As we have previously shown induction of TGF- β after stab wound injury in mice (Wang et al., 2007), we examined expression of T β RII to determine which cells in the cortex and hippocampus were able to respond to the induced TGF- β . The injury we used, stab wound injury (SWI) to the cerebral cortex, is a penetrating injury that induces strong local changes in inflammation and astrocytic responses (Wang et al., 2007). We found that T β RII was upregulated on NeuN+ neurons in the ipsilateral cortex (Fig. 1e, f) at 1 dpl in comparison to expression in the contralateral hemisphere (Fig. 1a, b). T β RII expression was induced in cortical layers III–VI and was located within the soma of neurons (Fig. 1f3–6). In the

ipsilateral hippocampus at 1 dpl, T β RII was upregulated specifically in CA1 neurons, in the cell soma (Fig. 1g, h, h1–6), while there were only sporadic T β RII expressing neurons in the contralateral CA1 region (Fig. 1c, d). At 1 dpl, T β RII was not widely expressed in astrocytes (data not shown), but its expression was induced in reactive astrocytes by 7 dpl (Fig. 4). T β RII was also expressed in astrocytes starting to form the glial scar at the border of the lesion at 7 dpl (Fig. 4a). These results suggest that neurons, by upregulating T β RII expression, are able to respond to TGF- β at early time points after injury. The astrocytic expression of T β RII occurred at later time points, suggesting a delayed astrocytic response to TGF- β in this model of traumatic brain injury (Table 1).

Smad3 expression is induced in cortical and hippocampal neurons after injury

To determine whether Smad3 may be involved in the neuronal response to TGF- β after injury, we examined Smad3 expression in the cortex and hippocampus at different time points post lesion. In the normal mouse brain, we found very low Smad3 expression in cortical and hippocampal neurons (data not shown). In the ipsilateral cortex Smad3 was strongly expressed in neurons throughout cortical layers III–VI (Fig. 2d, e–h, Table 1) at 1 and 3 dpl. However, Smad3 was mostly localized in the cell soma in these neurons, with only occasional nuclear immunoreactivity (Fig. 2f1–3). To determine whether activated Smad3 was localized in the nucleus we utilized an antibody against phosphorylated Smad3, pSmad3. Staining for pSmad3 showed mainly nuclear localization (Fig. 2n1–2). This staining was induced in the ipsilateral cortex at 1 dpl (Fig. 2l–n) indicating that injury initiates Smad3 phosphorylation and translocation to the nucleus. In contrast, in the contralateral hemisphere, Smad3 and pSmad3 staining was observed in only a few neurons (Fig. 2a–c, i–k). The intensity of Smad3 immunoreactivity was doubled in the ipsilateral cortex and hippocampus at 1 and 3 dpl, returning to baseline levels by 7 dpl (Figs. 2h and 3h). Interestingly, Smad3 immunoreactivity was also induced in the contralateral hippocampus at 1 dpl (Fig. 3g), but not at later time points (3 and 7 dpl, data not shown). Most Smad3+ cells also expressed T β RII in the ipsilateral cortex (Fig. 2o–q), with the percentage of colabelled Smad3+/T β RII+ cells increasing from 67% to 86% of the total Smad3+ cells from 1 to 14 dpl (Fig. 2r). The percentage of pSmad3+/T β RII+ cells also increased with time after injury, although at 1 dpl approximately 40% of pSmad3+ cells also expressed T β RII increasing to 93% of the total pSmad3+ cells by 7 dpl (Fig. 2w). Similarly, in the ipsilateral hippocampal CA1 region the vast majority of Smad3+ or pSmad3+ cells coexpressed T β RII, with the percentage of Smad3+/T β RII+ cells increasing with time after injury (Fig. 3r, w). Taken together these data suggest that TGF- β signaling through Smad3 in neurons is important to the early neuronal response to injury.

Smad3 expression in reactive astrocytes and glial scar after injury

Astrocytes respond to traumatic injury by becoming activated. If the injury is sufficiently severe, then these reactive astrocytes participate in many processes that lead to the formation of a glial scar (Sofroniew, 2009). We have previously shown that the absence of Smad3 in mice leads to reduced and more rapid scar formation implicating Smad3 signaling in the process of glial scar formation (Wang et al., 2007). In our stab wound injury model, we detect Smad3 and pSmad3 in GFAP+ reactive astrocytes in the cortex at 3 dpl with a peak expression at 7 dpl (Fig. 4, Table 1), corresponding with the degree of hypertrophy of the reactive astrocytes. Astrocytic Smad3 expression had almost disappeared by 14 dpl when the reactive astrocytes had the morphology of tangled processes and indistinguishable cell bodies forming part of the glial scar ((Wang et al., 2007) and data not shown). In our hands, Smad3 expression in astrocytes was undetectable by immunohistochemistry in the normal brain, in the contralateral

Fig. 1. Increase in *TβRII* expression in the ipsilateral (Ip) cortex and hippocampus after stab wound injury. (a–h) *TβRII* staining in the ipsilateral cortex (e, f) and hippocampus (g, h) in comparison to the contralateral cortex (a, b) and hippocampus (c, d). At 1 dpl, *TβRII* expression (red) is increased in neurons (NeuN, green) in the ipsilateral cortex (e, f) and hippocampal CA1 region (g, h). Higher power images are shown of the boxed area of the ipsilateral cortex (f1–2) and the hippocampal CA1 region (h1–2). Arrowhead in f1 points to cell shown in f3–f6. Arrowhead in h1 corresponds to cell shown in h3–h6. There is little detectable *TβRII* expression in the contralateral (Cl) cortex (cx) (a) or hippocampal CA1 (c) where the number of neurons is abundant (b and d). Scale Bars: 20 μm (f3–6, h3–h6), 50 μm (a–h, f1, f2, h1, h2).

hemisphere at all time points examined and at 1 dpl in the ipsilateral hemisphere (data not shown). *Smad3* expression in hippocampal astrocytes was also strongest at 7 dpl (Fig. 4) corresponding to the greatest GFAP expression. However, in the hippocampus even at 7 dpl *Smad3* was only expressed in a few astrocytes, and was weak at other

time points (Table 1). These data suggest that astrocytic induction of *Smad3* expression was delayed relative to that of neurons.

Smad3 deficiency decreases neuronal viability in the cortex and hippocampal CA1 region

To determine whether *Smad3* has a functional role in neuroprotection after injury, we compared neuronal viability after TBI between wild-type and *Smad3* null mice. Traumatic brain injury causes neuronal damage in cortical and hippocampal regions that can be determined by NeuN density (Collombet et al., 2006; Unal-Cevik et al., 2004). We therefore quantified the number and intensity of NeuN+ cells around the lesion core in injured mice and in the corresponding area in naïve (control) mice in both wild-type and *Smad3* null mice. Stab wound injury led to a reduction in NeuN cell number and intensity in wild-type and *Smad3* null mice (Fig. 5). However, the loss of NeuN staining was significantly greater in *Smad3* null mice than wild-type mice. After injury, at 1 dpl, NeuN+ cell number was reduced 28% in wild-type mice and approximately 60% in *Smad3* null mice as compared with the control wild-type and *Smad3* null mice respectively. At 7 dpl, the NeuN+ cell number was reduced approximately 45% in wild-type mice and about 75% in *Smad3* null mice (Fig. 5e). The intensity of NeuN immunoreactivity was also more strongly reduced in *Smad3* null mice in comparison to

Table 1
Colocalization of *TβRII*, *Smad3* and *pSmad3* with astrocytic and neuronal markers in the cortex and hippocampal CA1 region in control and injured brains at different times post lesion (dpl).

	Control		1 dpl		3 dpl		7 dpl		14 dpl	
	CX	CA1	CX	CA1	CX	CA1	CX	CA1	CX	CA1
<i>Neurons (NeuN +)</i>										
<i>TβRII</i>	–	–	+++	+++	+++	+++	++	++	+	+
<i>Smad3</i>	+	+	+++	+++	++	++	+	+	+	+
<i>pSmad3</i>	+/-	+/-	++	++	+	+	+	+	+/-	+/-
<i>Astrocytes (GFAP +)</i>										
<i>TβRII</i>	–	–	–	–	+	+	++	++	+	+
<i>Smad3</i>	–	–	–	–	+	+	++	++	+	+
<i>pSmad3</i>	–	–	–	–	+/-	+/-	+	+	+	+

The degree of colocalization is graded as:
–: negative ±: occasional +: low ++: intermediate +++: strong.

Fig. 2. Increase in Smad3 and pSmad3 expression and colocalization with TβRII in the cortex of wild-type mice after injury. Coronal sections were immunostained for either Smad3 or pSmad3 (red) together with either a neuronal (NeuN) marker or the TGF-β receptor, TβRII (green). (a–f) At 1 day post lesion (dpl), there was decreased neuronal density in the ipsilateral (Ip) cortex (d) in comparison to that in the contralateral (Cl) cortex (a). Immunostaining for Smad3 was dramatically increased in the ipsilateral cortex (e) around the injury site, predominantly in NeuN+ neurons (merged images f, and high magnification f1–f3 with orthogonal projections in f3). (i–n) pSmad3 staining was increased in the ipsilateral cortex specifically in neuronal nuclei as compared to the mainly cytoplasmic neuronal staining of total Smad3 (compare n to f). (g) Quantification of Smad3 expression in the ipsilateral or contralateral cortex at 1 dpl, relative to that in control mice. (h) Time course of intensity of Smad3 expression in the ipsilateral cortex shows that the peak expression occurred at 1 and 3 dpl (mean ± SEM, $n = 6$), **** $p < 0.0001$; *** $p < 0.001$ compared to control. (o–v) TβRII colocalizes in cells expressing Smad3 (o–q) and pSmad3 (s–v) in the ipsilateral cortex at 1 dpl. (r, w) Quantification of the cells expressing TβRII together with Smad3 (r) or pSmad3 (w) as a percentage of the total number of Smad3 or pSmad3 expressing cells. Scale Bars: 20 μm (f1–3, n1–2), and 50 μm (a–v).

wild-type mice. NeuN immunoreactivity was reduced only at 1 dpl (approximately 30%) in wild-type mice, but reduced at 1 and 3 dpl (about 70% reduction at 1 dpl and 30% reduction at 3 dpl) in Smad3 null mice (Fig. 5f). There was no difference in either the NeuN+ cell number or intensity between control wild-type and Smad3 null mice (Fig. 5e, f),

nor did we find any obvious change of NeuN immunoreactivity in the contralateral cortex (data not shown).

In the hippocampus, Smad3 null mice displayed a dramatic reduction of viable neurons after stab wound injury with almost a complete disappearance of NeuN immunoreactivity at 1 dpl and a

Fig. 3. Increase in Smad3 and pSmad3 expression and colocalization with TβRII in the hippocampus of wild-type mice after injury. Coronal sections were immunostained for either Smad3 or pSmad3 (red) together with either a neuronal (NeuN) marker or the TGF-β receptor, TβRII (green). (a–f) At 1 day post lesion (dpl), there was decreased NeuN staining (green) in the ipsilateral CA1 (d) in comparison to the contralateral CA1 (a). Smad3 staining colocalized with neuronal staining in the soma (merged image in f), although some neurons displayed nuclear Smad3 expression (high magnification in f1–3, and orthogonal projection in f3). (g) Intensity of Smad3 staining at 1 dpl showing elevated Smad3 expression in the ipsilateral and contralateral CA1. (h) Time-course of intensity of Smad3 expression (mean ± SEM, $n = 6$), **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$ compared to control. (i–n) At 1 dpl pSmad3 staining was increased in the ipsilateral CA1 specifically in neuronal nuclei as compared to the mainly cytoplasmic neuronal staining of total Smad3 (compare n to f). Arrowhead in n corresponds to cell shown in n1. (o–q) In the ipsilateral CA1, TβRII colocalizes in cells expressing Smad3 at 1 dpl (high magnification in q1). (s) Some TβRII/pSmad3 colocalization is still detectable at 14 dpl. Arrowhead in s corresponds to cell shown in s1–3. (r, t) Quantification of TβRII expression together with Smad3 (r) or pSmad3 (t) as a percentage of the total number of Smad3 or pSmad3 expressing cells. Scale Bars: 20 μm (f1–3, n1, q1, s1), and 50 μm (a–s).

reduction at 3 and 7 dpl in the ipsilateral CA1 area (Fig. 6). Wild-type mice showed a significant, but far smaller decrease in both NeuN number and intensity in the ipsilateral CA1 only at 1 dpl (Fig. 6i, j). In

the contralateral CA1, Smad3 null mice showed reduced NeuN immunoreactivity only at 1 dpl, with no decrease in the number of NeuN+ cells (Fig. 6e, f, j). Smad3 null mice did not show any reduction

Fig. 4. Astrocytic expression of *TβRII* and *Smad3* in wild-type mice at 7 days post lesion. (a) Astrocytic *TβRII* (red) expression colocalizes with GFAP + reactive astrocytes (green) in the ipsilateral cortex. Circle in a is shown in inset in a1 and a2. Arrows in a show the beginning of the formation of glial scar. (b) *Smad3* expression is localized to some reactive astrocytes in the injured cortex. Strong cytoplasmic *Smad3* (b, b1–4) and nuclear p*Smad3* (c, c1–4) immunoreactivity is detected in astrocytes at the lesion border (c). Circled cells are shown in higher magnification in adjacent panels. *Smad3* (d, d1) and p*Smad3* (e, e1) were also localized in reactive astrocytes (GFAP) in the ipsilateral hippocampus. Scale Bars: 50 μm (a, b, b1, c, c1, d, e) and 20 μm (a1–2, b2–4, c2–4, d1, e1).

in NeuN + cell numbers in the ipsilateral or contralateral CA3 or DG regions (data not shown). Early loss of NeuN immunoreactivity could be due to damage to neurons that subsequently recover (Collombet et al., 2006; Unal-Cevik et al., 2004). We therefore examined Nissl staining of sections taken from mice at 7 dpl. This staining confirmed significant neuronal loss in the ipsilateral CA1 region of *Smad3* null mice, with a less pronounced but still detectable loss in this region in wild-type mice (Fig. 6c, d, g, h). There were also slightly reduced neuronal numbers in the ipsilateral cortex only in the *Smad3* null mice (data not shown). However, cresyl-violet staining did not reveal any neuronal loss in the contralateral CA1 in *Smad3* null mice in comparison to wild-type mice (Fig. 6g, h). These data suggest that, after injury, *Smad3* deficiency leads to increased neuronal death in the ipsilateral cortex and hippocampus close to the cortical lesion site.

Absence of *Smad3* protein results in increased neuronal degeneration after injury

We next determined whether *Smad3* deficiency altered the number of neurodegenerative neurons in the cortex and hippocampus after stab wound injury by examining Fluoro-Jade B staining in wild-type and *Smad3* null mice. Fluoro-Jade B is a specific fluorescent marker

for degenerating neurons (Schmued et al., 1997; Schmued and Hopkins, 2000) and has been widely used to detect neuronal degeneration induced by different types of insults (Moreira et al., 2007; Ribba-Bosch and Perez-Clausell, 2004; Sun et al., 2007). In wild-type mice stab wound injury caused significant Fluoro-Jade B staining in the ipsilateral cortex (Fig. 7) and hippocampal CA1 region (Fig. 8) at 1 and 3 dpl. However, in sections taken from *Smad3* null mice there were significantly more Fluoro-Jade B labeled degenerating neurons in both cortex and CA1 at both 1 and 3 dpl (Figs. 7 and 8). These data show that the absence of *Smad3* led to increased neuronal degeneration after injury. No neuronal degeneration was detected in the CA3 region or the dentate gyrus (DG) of the hippocampus (data not shown). We also found no Fluoro-Jade B staining in the contralateral cortex or hippocampus at any time point after injury (data not shown). To determine whether the neurons were dying by apoptosis, we assessed cell death using a TUNEL assay (to measure nuclear DNA fragmentation) in the injured cortex of both wild-type and *Smad3* null mice. We co-stained with NeuN to show that these dying cells were predominantly neurons. We detected a clear increase in TUNEL + cells in the ipsilateral cortex surrounding the lesion of wild-type mice (Fig. 7f). However, there were clearly more TUNEL + cells in the ipsilateral cortex (Fig. 7f, g) and CA1 region (Fig. 8h, i) of *Smad3* null mice in comparison to wild-type mice. TUNEL + cells displayed typical

Fig. 5. Loss of viable neurons in the injured cortex of *Smad3* deficient mice. Attenuated neuronal density (NeuN, in green) around the lesion core in the cortex was observed in *Smad3* null mice (—/—) compared to wild-type (+/+) mice at 1 day post lesion (dpl) (a, a1, b, b1) and 7 dpl (c, c1, d, d1). (e) No difference was found between genotypes in the number of neurons (NeuN+ cells) in control mice but a significant reduction in the number of neurons was found at 1, 3 and 7 dpl in *Smad3* null mice. (f) The intensity of NeuN staining was decreased in wild-type mice only at 1 dpl, but at 1 and 3 dpl in *Smad3* null mice. At 1 and 3 dpl *Smad3* null mice showed a lower NeuN intensity than wild-type mice (mean $\pm$ SEM, $n = 6$); *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to control wild-type mice; ##### $p < 0.0001$, ## $p < 0.01$ compared to control *Smad3* null mice; +++++ $p < 0.0001$, + $p < 0.05$ compared to injured wild-type mice. Scale Bars: 100 μ m (a–d), 50 μ m for (a1, b1, c1, d1).

apoptotic morphology showing apoptotic bodies and condensed chromatin (Fig. 7g1–3). The involvement of the caspase pathway was demonstrated with the colocalization of cleaved caspase-3

(Fig. 7i1–3). Thus, our data show that *Smad3* ablation led to increased neuronal death by apoptosis in the ipsilateral cortex and hippocampus at early time points after stab wound injury.

Fig. 6. Loss of viable neurons in the injured hippocampal CA1 region of Smad3 deficient mice. (a,b) A dramatic decrease in NeuN immunoreactivity in the ipsilateral CA1 area was found in Smad3 null mice (–/–) compared to wild-type (+/+) mice at 1 day post lesion (dpl) (also shown in i). (j) The intensity of NeuN staining in Smad3 null mice in the ipsilateral CA1 was significantly different from wild-type mice at 1, 3 and 7 dpl but not in control mice. In the contralateral CA1, Smad3 null mice had reduced NeuN intensity only at 1 dpl. (mean $\pm$ SEM, $n = 5-6$). ** $p < 0.01$ compared to control wild-type mice; **** $p < 0.0001$, *** $p < 0.001$ compared to control Smad3 null mice; **** $p < 0.0001$, **** $p < 0.001$ compared to injured wild-type mice. (c–h) Cresyl-violet staining of 7 dpl hippocampi showed a reduced neuronal density in the ipsilateral CA1 region in both genotypes, although a more pronounced reduction in Smad3 null mice (d). Scale Bars: 50 μ m (a, b, e, f) and 400 μ m (c, d, g, h).

Discussion

After stab wound injury, mice that do not express the TGF- β -regulated transcription factor Smad3, show greater loss of neurons in the cerebral cortex as well as the CA1 area of the hippocampus compared to wild-type mice. This increased neuronal damage is manifest during the acute injury phase (1 and 3 dpl), which coincides with increased neuronal expression of the TGF- β type II receptor, T β RII, in wild-type mice. Smad3 is also induced in neurons at these early time points after injury in wild-type mice. Interestingly, astrocytic expression of T β RII and Smad3 protein was strongly induced in the ipsilateral cortex and CA1 region of the hippocampus only at a later time point, by 7 dpl. Taken together, our results suggest that TGF- β related cytokines signaling through Smad3 protect neurons in the damaged cortex and hippocampus during the acute injury response.

Smad3 signaling involved in neuroprotection may be initiated by TGF- β

Several lines of evidence demonstrate that TGF- β is a potent neuroprotective agent. TGF- β protects neurons in many models of neurodegenerative diseases, e.g. amyotrophic lateral sclerosis (ALS) (Day et al., 2005), Huntington's disease (Battaglia et al., 2011), Alzheimer's disease (Caraci et al., 2011), Parkinson's disease (Gonzalez-Aparicio et al., 2010) and status epilepticus (Li et al., 2013). TGF- β 1 knock-out mice show prominent neuronal cell death in various parts of the brain (Brionne et al., 2003), and genetic reduction of TGF- β 2 leads to a loss of dopaminergic neurons in mice (Andrews et al., 2006).

Cell culture studies have also shown that TGF- β signaling is required for maintaining neuronal survival after various insults (Bruno et al., 1998; Walshe et al., 2011; Zhu et al., 2002). Inhibition of TGF- β signaling specifically in neurons leads to increased neurodegeneration (Tesseur et al., 2006). Our data show that ablation of Smad3, a critical mediator of TGF- β signaling, caused significantly more neuronal loss after injury. The time course of neuronal death correlated with the timing of elevated neuronal Smad3 expression in wild-type mice. It has also previously been shown that TGF- β 1 is strongly elevated acutely after traumatic injury in mice (Chen et al., 2011). Thus, there is strong evidence that TGF- β signaling through Smad3 may be promoting the suggested neuroprotection in this injury model. However, other TGF- β superfamily cytokines including activins, nodal and GDFs can also initiate Smad3 signaling (Massague et al., 2005), and activin has neuroprotective properties in vitro and in vivo (Sulyok et al., 2004; Tsuchida et al., 2009). These related cytokines differ in the receptors that they use to activate Smad3. Activin, nodal and GDF11 utilize Activin Receptor IIB as their ligand binding subunit, which complexes together with type I receptors (ALK-4, ALK-7 and ALK-5 respectively) to form a functional signaling unit (Andersson et al., 2006; Kumar et al., 2001; Labbe et al., 1998). TGF- β is the only known cytokine to signal through T β RII (Massague, 2000). Thus, although we cannot exclude the role of TGF- β related cytokines in contributing to the effects we report, the co-expression of T β RII and Smad3 in the majority of Smad3+ cells (Figs. 2 and 3), together with the known effects of TGF- β on neuroprotection imply that TGF- β signaling through Smad3 may be a major regulator of neuroprotection after stab injury.

Fig. 7. Cortical neuronal death is increased in *Smad3* deficient mice after brain injury. (a–d) Increased numbers of degenerating neurons indicated by Fluoro-Jade B (FJB) staining (in green) around the cortical impact site in *Smad3* null mice (–/–) compared to wild-type mice (+/+) at 1 and 3 days post lesion (dpl) (a1–d1 are enlargements of the boxed areas in their respective figures). These data are shown graphically in (e). (f, g) Increased TUNEL staining in *Smad3* null mice confirmed the increased neuronal death in these mice. High magnification of the square area in (g) shows TUNEL + neurons (g1 in green), NeuN (g2 in red) and colocalization (g3). Pro-apoptotic cleaved caspase-3 (i1 in green) was localized in the nucleus of neurons (i2, NeuN in red, i3 merged image) within the cortical site of the injury. Arrowheads indicate the extent of degenerating neurons (a–d, f, g). (mean ± SEM, n = 6), *****p* < 0.0001; **p* < 0.05 compared to injured wild-type mice. Scale Bars: 20 μm (g1–g3, i1–i3), 150 μm (a1–d1), 300 μm (f, g), and 500 μm (a–d).

Mechanism of TGF-β mediated neuroprotection

The mechanisms through which *Smad3* can mediate neuroprotection are not clear. TGF-β activation of *Smad3* is through activation of the kinase activity of ALK-5, TβR1. However, it has previously been shown that TGF-β mediated neuroprotection of cultured cortical or hippocampal neurons is through activation of ALK1-NF-κB anti-apoptotic signaling (Konig et al., 2005). Our data suggest that *Smad3* is also capable of promoting neuroprotection. Indeed, a different line of *Smad3* null mice have reduced neuronal survival of dopaminergic neurons (Tapia-Gonzalez et al., 2011). These mice also have reduced phosphorylated Erk in dopaminergic neurons but not in other brain areas, suggesting that there may be specific interactions between *Smad3* and Erk signaling in stressed neurons. Inhibition of *Smad3* in neuroblastoma cells, through transfection of dominant negative *Smad3*, also leads to reduced cell survival, while constitutively active *Smad3* can rescue the decrease in cell viability caused by decreased TGF-β receptor signaling (Tesseur et al., 2006). Thus, taken together there is significant evidence that TGF-β signaling through *Smad3* enhances neuronal survival.

Hippocampal neuronal loss after stab wound injury

We detected dramatic neuronal degeneration in the hippocampal CA1 area ipsilateral to the injury side though our brain injury has no direct injury to the hippocampus (Wang et al., 2007). Previous reports have

shown that a controlled cortical impact injury can also cause a significant neuronal loss in the CA1 and CA3 regions of the hippocampus (Anderson et al., 2005; Zhao et al., 2012). Hippocampal neurons are extremely vulnerable to different types of insults (Bonner et al., 2010; Tsuchiya et al., 2011). However, after stab wound injury we found little neuronal cell death in the dentate gyrus of the hippocampus (data not shown), in disagreement with other injury models which resulted in significant neuronal death in this region (Zhao et al., 2012). It has previously been suggested that the most vulnerable neurons of the hippocampus are the immature neurons of the subgranular zone of the dentate gyrus (Gao and Chen, 2009), which we did not find to be affected by this injury. Thus, there does seem to be differential vulnerability of specific neuronal populations to different models of injury.

Absence of *Smad3* makes the contralateral hippocampal CA1 neurons damaged after stab wound injury

Interestingly, we observed that NeuN immunoreactivity in the contralateral CA1 was significantly reduced in *Smad3* null mice compared to wild-type mice at 1 dpl (Fig. 5j). This difference was no longer detectable by 3 dpl. Multiple studies have demonstrated that the early loss of NeuN immunoreactivity, a specific marker of mature neurons, can be used to determine reversible neuronal damage (Collombet et al., 2006; Unal-Cevik et al., 2004). NeuN immunoreactivity relies on the antigenicity of the NeuN antigen that can be altered after injury, but

Fig. 8. Hippocampal neuronal death is increased in *Smad3* deficient mice after brain injury. Increased numbers of degenerating neurons indicated by Fluoro-Jade B (FJB) staining (in green) in the hippocampus of *Smad3* null mice (–/–) compared to wild-type mice (+/+) at 1 and 3 days post lesion (dpl). These data are shown graphically in (g). (mean ± SEM, $n = 6$), *** $p < 0.001$; * $p < 0.05$, compared to injured wild-type mice. An increase in apoptotic neurons (TUNEL + cells in green, and NeuN in red) was detected in the CA1 region in the *Smad3* null mice (–/–) (i) compared to wild-type *Smad3* mice (+/+) (h) at 1 dpl. Scale bar = 20 μm (h, i) and 100 μm (a–f).

neurons with reduced NeuN immunoreactivity may still be able to recover. Our finding of reduced NeuN immunoreactivity at 1 dpl in *Smad3* null mice but not at 3 dpl indicates that these neurons in the hippocampal CA1 contralateral to the injury were damaged but not destroyed. Our data therefore highlight the injury-induced enhanced vulnerability of this neuronal population in the contralateral hippocampus and show that *Smad3* signaling in these neurons is neuroprotective.

Does activation of Smad3 in glial cells contribute to the neuroprotective effect of Smad3?

Injury induces neuronal *Smad3* expression in the ipsilateral cortex and hippocampus, at early time points after injury in wild-type mice

(Figs. 2 and 3). Astrocytic expression of *Smad3* is not detectable until 3 dpl. As neuronal degeneration peaks at 1 dpl, it is likely that *Smad3* expression in neurons is directly neuroprotective at early time points. There are more than twice as many degenerating neurons in *Smad3* null mice than in wild-type mice at 1 and 3 dpl, suggesting that the lack of *Smad3* specifically in neurons is detrimental for neuronal survival. The lack of an early astrocytic *Smad3* response is in agreement with our previously report that there was no difference between wild-type and *Smad3* null mice in the number of GFAP+ astrocytes at 3 dpl, but a significant difference at 7 dpl (Wang et al., 2007). Thus, at later time points after stab wound injury, *Smad3* signaling within astrocytes may contribute to a non-cell autonomous effect on neuronal survival. Indeed, *Smad3* signaling in cultured astrocytes is known to be important for specific astroglial responses that support neuronal

survival, such as up-regulation of plasminogen activator inhibitor type 1 (PAI-1) (Docagne et al., 2002). Smad3 action in astrocytes is also important in promoting expression of genes and protein that are critical for glial scar formation such as induction of some chondroitin sulfate proteoglycan genes (Susarla et al., 2011). Therefore, the delayed elevation of Smad3 expression in astrocytes may be more important for promotion of reactive gliosis leading to glial scar formation rather than the promotion of neuron survival after injury.

We have previously shown that the Smad3 null mice respond to stab wound injury with reduced glial scar formation, and less infiltration of neutrophils and macrophages (Wang et al., 2007). We show here however, increased neuronal degeneration and death in the ipsilateral cortex and hippocampus in these Smad3 mice after the same injury. Our data point once again to the complex reactions that TGF- β or related cytokines can elicit, and the necessity of understanding all of the downstream actions of these context dependent cytokines. Initially, we sought to differentiate beneficial from detrimental effects through delineating specific intracellular signaling pathways. However, our data show that Smad3 signaling may mediate both neuronal survival and promotion of scar formation. As these mice are complete knockouts, it is still possible, that cell-specific conditional inducible knockouts may be able to show beneficial effects through deletion of Smad3 in only one cell type in the adult. Our data suggest that caution is required in utilizing any therapeutic that seeks to globally inhibit TGF- β signaling within the CNS.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.expneurol.2013.10.008>.

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