

BRAIN COMMUNICATIONS

Activation of both transforming growth factor- β and bone morphogenetic protein signalling pathways upon traumatic brain injury restrains pro-inflammatory and boosts tissue reparatory responses of reactive astrocytes and microglia

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Various ligands and receptors of the transforming growth factor- β superfamily have been found upregulated following traumatic brain injury; however, the role of this signalling system in brain injury pathophysiology is not fully characterized. To address this, we utilized an acute stab wound brain injury model to demonstrate that hallmarks of transforming growth factor- β superfamily system activation, such as levels of phosphorylated Smads, ligands and target genes for both transforming growth factor- β and bone morphogenetic protein pathways, were upregulated within injured tissues. Using a bone morphogenetic protein-responsive reporter mouse model, we showed that activation of the bone morphogenetic protein signalling pathway involves primarily astrocytes that demarcate the wound area. Insights regarding the potential role of transforming growth factor- β superfamily activation in glia cells within the injured tissues were obtained indirectly by treating purified reactive astrocytes and microglia with bone morphogenetic protein-4 or transforming growth factor- β 1 and characterizing changes in their transcriptional profiles. Astrocytes responded to both ligands with considerably overlapping profiles, whereas, microglia responded selectively to transforming growth factor- β 1. Novel pathways, crucial for repair of tissue-injury and blood-brain barrier, such as activation of cholesterol biosynthesis and transport, production of axonal guidance and extracellular matrix components were upregulated by transforming growth factor- β 1 and/or bone morphogenetic protein-4 in astrocytes. Moreover, both ligands in astrocytes and transforming growth factor- β 1 in microglia shifted the phenotype of reactive glia cells towards the anti-inflammatory and tissue reparatory 'A2'-like and 'M0/M2'-like phenotypes, respectively. Increased expression of selected key components of the *in vitro* modulated pathways and markers of 'A2'-like astrocytes was confirmed within the wound area, suggesting that these processes could also be modulated *in situ* by the integrated action of transforming growth factor- β and/or bone morphogenetic protein-mediated signalling. Collectively, our study provides a comprehensive comparative analysis of transforming growth factor- β superfamily signalling in reactive astrocytes and microglia and points towards a crucial role of both transforming growth factor- β and bone morphogenetic protein pathways in modulating the inflammatory and brain injury reparatory functions of activated glia cells.

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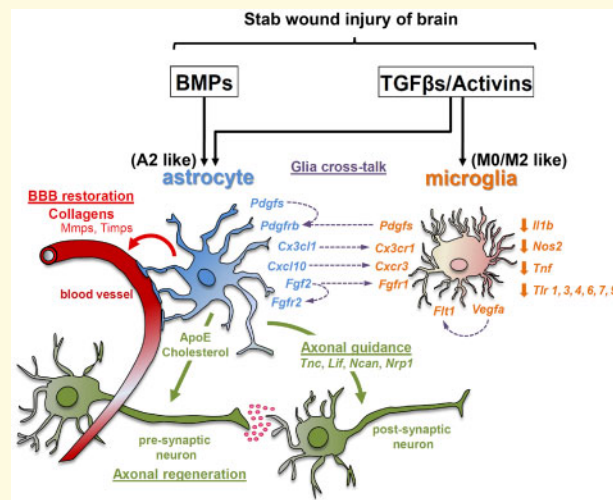
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Abbreviations: ALK = anaplastic lymphoma kinase; BMP = Bone morphogenetic protein; BRE = BMP-responsive elements; dpi = days post-injury; eGFP = enhanced green fluorescent protein; ERK = extracellular signal-regulated kinase; FPKM = fragments per kilobase million; GLAST = glutamate-aspartate transporter; GO = gene ontology; IPA = ingenuity pathway analysis; NRF2 = nuclear factor erythroid 2-related factor 2; PDL = poly-D-lysine; PDGF = platelet-derived growth factor; PI3K = phosphatidylinositol-3-kinase; qRT-PCR = quantitative real-time polymerase chain reaction; SW = stab wound; VEGF = vascular endothelial growth factor; TBI = traumatic brain injury; TGF β = transforming growth factor beta

Graphical Abstract



Introduction

Traumatic brain injury (TBI), defined as alteration in brain function provoked by external mechanical force (Menon *et al.*, 2010), is the main cause of injury-related death, disability and mental disorders, thus representing major public health issue (Majdan *et al.*, 2016; Dewan *et al.*, 2018). According to recent epidemiological studies, TBI affects annually more than 60 million individuals, justifiably characterized as 'silent epidemic' (Maas *et al.*, 2017; Dewan *et al.*, 2018).

TBI pathophysiology is characterized by acute necrotic or delayed apoptotic neuronal death, cytokine and chemokine production, infiltration of peripheral immune cells and activation of astrocytes and microglia (Helmy *et al.*, 2011; Burda *et al.*, 2016; Jassam *et al.*, 2017). Astrocytes play important roles in the CNS providing structural support, maintaining blood–brain barrier integrity, regulating the neuronal microenvironment through clearance of excessive neurotransmitters (e.g. glutamate and GABA), promoting formation and function of synapses, pruning synapses by phagocytosis and secreting a variety of trophic and growth factors (Sofroniew and Vinters, 2010; Chung *et al.*, 2013; Bylicky *et al.*, 2018). Due to blood–

brain barrier restriction in lipoprotein exchange, astrocytes are the main producers and exclusive providers of cholesterol in the adult brain (Pfrieger and Ungerer, 2011; Sidoryk-Wegrzynowicz *et al.*, 2011).

In pathological situations astrocytes undergo dramatic transformation, become 'reactive' and depending on the conditions acquire neurotoxic or neuroprotective phenotypes (Myer *et al.*, 2006; Burda *et al.*, 2016; Bylicky *et al.*, 2018). Following a terminology that parallels the 'M1' and 'M2' macrophage nomenclature (Biswas and Mantovani, 2010; Sica and Mantovani, 2012), neurotoxic/pro-inflammatory astrocytes, generated by inflammatory insult, are named 'A1', whereas, neuroprotective/anti-inflammatory astrocytes known as 'A2' are induced in the context of various types of brain injury such as ischaemic damage and produce neurotrophic and tissue-repair factors (Zamanian *et al.*, 2012; Liddelow *et al.*, 2017). The two types of astrocytes can be distinguished on the basis of functionality and characteristic gene expression profiles (Zamanian *et al.*, 2012; Liddelow *et al.*, 2017).

Microglia are heterogeneous resident immune cells within the CNS, surveying the brain parenchyma and reacting to any insult that disrupts homeostasis (Szepesi *et al.*, 2018). Like astrocytes, microglia upon activation, and depending

on the context, acquire pro-inflammatory/neurotoxic or anti-inflammatory/neuroprotective phenotypes. In line with the 'M1'/'M2' macrophage nomenclature, the two microglial phenotypes are frequently referred to as 'M1'- and 'M2'-microglia, respectively (Fumagalli *et al.*, 2011; Michell-Robinson *et al.*, 2015; Loane and Kumar, 2016). In the context of neuropathology, cross-talk between microglia and astrocytes is critical for coordination of inflammation and tissue-injury repair (Karve *et al.*, 2016; Donat *et al.*, 2017). Pertinently, recent studies showed that reactive inflammatory microglia guide astrocytes towards the 'A1' phenotype (Liddelow *et al.*, 2017) and, conversely, earlier studies demonstrated that activated astrocytes can exert inhibitory effects on microglial activation (Gao *et al.*, 2013).

Among the molecular systems implicated in TBI pathophysiology is the transforming growth factor- β (TGF β)-superfamily (Schachtrup *et al.*, 2010; Chen *et al.*, 2018), which encompasses structurally related polypeptides including, among others, TGF β s, activins and bone morphogenetic proteins (BMPs; Shi and Massague, 2003). TGF β -superfamily members signal via hetero-tetrameric complexes of type-II and type-I receptors, which possess Ser/Thr kinase activity and activate 'canonical' and 'non-canonical' pathways. Canonical pathways involve phosphorylation-dependent activation of downstream effectors, the receptor-regulated Smads (R-Smads), which are organized into two branches, the TGF β /Activin and BMP branches that involve Smads 2/3 or Smads 1/5/8, respectively (Shi and Massague, 2003). Phosphorylated R-Smads form complexes with the 'common' Smad 4, enter the nucleus and regulate gene expression (Moustakas *et al.*, 2001; Budi *et al.*, 2017). Activation of canonical pathways up-regulates Smads 6 and 7, which function as negative feedback regulators (Miyazawa and Miyazono, 2017). 'Non-canonical' pathways are activated in parallel and involve pathways such as mitogen-activated protein kinases (MAPKs), Rho-like GTPases and phosphatidylinositol-3-kinase (PI3K)/AKT (Moustakas and Heldin, 2005; Zhang, 2009). Given the widespread expression of TGF β /Activin and/or BMP receptors within a tissue, even on the surface of the same cell, and the concurrent production of numerous ligands in health and disease (Rosendahl *et al.*, 2001, 2002), it is likely that the final response must depend on the integration of all generated signals and be influenced by the relative, quantitative and/or qualitative, characteristics of the activated canonical and/or non-canonical pathways. Therefore, besides the selective analysis of individual components of the TGF β -superfamily signalling system, approaches that analyse a broader spectrum of signalling events and components could unveil important functional properties of the system.

Several components of the TGF β -superfamily system, including ligands (TGF β 1, BMP4, BMP10) or receptors (TGFBR1, TGFBR2, ACVR1, BMPR2), are upregulated, either at protein and/or mRNA levels, in cells surrounding the trauma or in adult neurogenic niches following

TBI in rodents (Lindholm *et al.*, 1992; Lewen *et al.*, 1997; Huang *et al.*, 2010; Komuta *et al.*, 2010; Schachtrup *et al.*, 2010; Logan *et al.*, 2013; Chen *et al.*, 2018). Moreover, increased TGF β 1 and TGF β 2 protein levels have been described in human spinal cord injuries (Buss *et al.*, 2008), and BMP7 mRNA (Setoguchi *et al.*, 2001) and protein levels in rat spinal cord injury (Hampton *et al.*, 2007). Despite accumulated evidence, the precise role of this signalling system, in particular, the interplay between the TGF β and BMP branches in the context of TBI pathophysiology is still not fully understood. To address this, we have utilized the most controlled form of TBI, namely, the acute stab wound (SW) brain injury to carry out a comprehensive analysis of the TGF β -superfamily system, identify putative cellular targets within the wound area and characterize their response to TGF β and/or BMP. Our study demonstrates that both TGF β and BMP pathways are activated locally upon SW injury and show that astrocytes demarcating the wound area are the selective target of BMP signalling. By analysing *in vitro* treated 'reactive' astrocytes and microglia, we unveil novel processes critical for restoration of homeostasis that may be regulated by TGF β and/or BMP. Moreover, we provide evidence suggesting that both TGF β 1 and BMP4 modulate the plasticity of reactive astrocytes towards an 'A2'-like, anti-inflammatory phenotype, whereas TGF β 1 modulates the plasticity of activated microglia towards 'M0/M2'-like, quiescent and anti-inflammatory phenotypes, respectively. As such, our findings present a parallel analysis of the two signalling branches of the TGF β -superfamily system in the context of acute SW injury and provide insights that could aid the further clarification of their role and prospectively the design of novel therapeutic strategies.

Materials and methods

Extended version is available in the [Supplementary material](#).

Animals

Wild-type and BMP-responsive eGFP-expressing (BRE-eGFP) mice (Monteiro *et al.*, 2008), in C57BL/6 background, were maintained in individually ventilated cages with free access to food and water, under 12 h light/dark cycle at the animal facility of BRFAA. All experimental procedures performed were approved by Institutional Ethics Committee for Use of Laboratory Animals and the Greek Ministry of Agriculture.

Surgical procedure of SW injury

Males 3–4 months old were anaesthetized with 2–3% isoflurane and positioned in a stereotaxic frame (Kopf Instruments, USA), as previously described (Xilouri *et al.*, 2012). A unilateral lesion was performed to the right

hemisphere of the cerebral cortex by inserting a 19-gauge needle at the following coordinates: +0.6 mm anteroposterior, −1.6 mm mediolateral and −3.2 mm dorsoventral from the bregma as previously described (Buffo *et al.*, 2005; Frik *et al.*, 2018). The needle was twisted manually and gently retracted. This was repeated thrice in total and the skin was thereafter sutured.

Immunohistochemistry

Brain tissues were perfused intracardially with 4% paraformaldehyde and processed thereafter for immunohistochemical analysis, as previously reported (Xilouri *et al.*, 2012) with modifications described in [Supplementary material](#).

Preparation and maintenance of mixed glia cultures

Mixed glia cultures were prepared from P1 to P3 neonatal mice as previously described (McCarthy and de Vellis, 1980; Saura *et al.*, 2003), with modifications described in [Supplementary material](#). Cells utilized in the present study were grown in culture for a total of ~20–24 days before addition of recombinant TGFβ and/or BMP.

Fluorescence-activated cell sorting of cultured glia cells

Mixed glia cultures were harvested and cells were stained with antibodies against GLAST and CD11b proteins ([Supplementary Table 1](#)) for 45 min. Unstained samples and isotypic controls were also included. DAPI was added the last 5 min of incubation for dead-cell exclusion. Cells were sorted with a BD-FACSAriaTM-IIu as described in [Supplementary material](#).

In vitro stimulation and immunofluorescence analysis of cultured glia cells

Mixed glia, purified astrocytes and microglia, grown for ~20–24 days in total, were cultured in serum-free DMEM 1% Pen/Strep for the last 24 h before treatment with recombinant mouse TGFβ1, BMP4, Noggin or the SB431542 inhibitor, in the absence of FBS. Cells and culture media were collected by centrifugation (2000 rpm, RT, 2 min), snap frozen and stored at −80°C until use. Cells were cultured on poly-D-lysine (PDL)-coated coverslips for immunocytochemistry, as described in [Supplementary material](#).

Western blot and quantitative real-time PCR analysis

For protein or RNA isolation from TBI-subjected animals, isolated brains were placed on an acrylic mouse brain matrix and 2 mm coronal-sections were prepared. Tissue areas encompassing the trauma or the respective

contralateral/healthy area were further micro-dissected ([Fig. 1A](#)), snap frozen and stored at −80°C. For protein isolation, micro-dissected regions or primary glial cells were homogenized in RIPA buffer supplemented with protease and phosphatase inhibitors and western blot analysis was performed as previously reported (Xilouri *et al.*, 2012), with modifications described in [Supplementary material](#). Total RNA was isolated using Trizol and cDNA was synthesized as previously described (Sountoulidis *et al.*, 2012). The primer pairs and the amplification parameters used for quantitative real-time PCR (qRT-PCR) are shown in [Supplementary material](#).

Transcriptomic analysis

One microgram purified RNA was used for preparation of each cDNA library, using the TruSeq RNA Preparation Kit (Illumina), according to manufacturer's instructions. Library quality was evaluated with Agilent DNA Kit and concentration was measured using library-standards (KAPA Biosystems). Sequencing was performed at the Genome Center Facility of BRFAA. Bioinformatics analysis was performed using Ingenuity[®] Pathway Analysis (IPA[®], Qiagen), MultiExperiment Viewer (MeV, TM4 Microarray Software Suite; Saeed *et al.*, 2003), Venny 2.1 and Venn Diagram software (Pacific Northwest National Laboratory, U.S. Department of Energy).

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Data were analysed using one-way analysis of variance with Bonferroni's *post hoc* test analysis. One asterisk (*) corresponds to statistical significance of $P < 0.05$, two asterisks (**) to $P < 0.01$, three asterisks (***) to $P < 0.001$ and non-significant (ns) to $P > 0.05$. In few indicated cases, the Newman–Keuls Multiple Comparison Test was applied with one hashtag (#) corresponding to statistical significance of $P < 0.05$. In [Fig. 4B](#), differences were assessed using two-tailed unpaired *t*-test ($*P < 0.05$, $**P < 0.01$ and $***P < 0.001$). All analyses were performed using GraphPad Prism software version 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

Data availability

Raw data supporting the findings of this study are available from the corresponding author on request. Gene expression data are available from Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>), accession number PRJNA527753.

All materials used in this study are listed in [Supplementary Table 3](#).

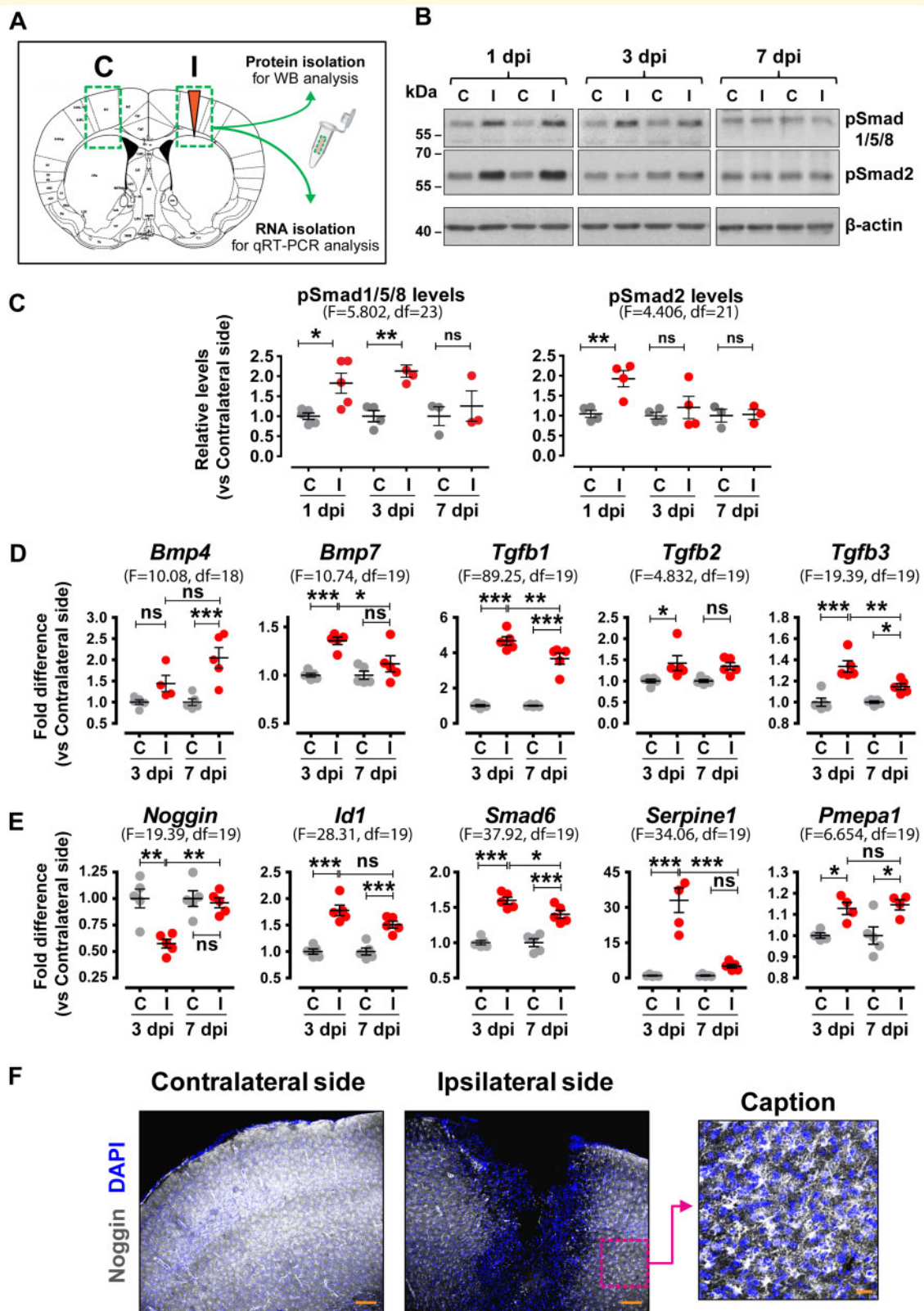


Figure 1 Increased levels of phosphorylated Smads, TGF β -superfamily ligands and relevant target genes are found within the TBI wound area. **(A)** Schematic representation of the strategy followed for tissue dissection of ipsilateral injured areas (I) and corresponding contralateral control areas (C) for protein or RNA isolation. **(B)** Representative immunoblots for pSmad1/5/8, pSmad2 and β -actin (loading control) levels in protein extracts from dissected contralateral (C) and ipsilateral (I) tissues at 1, 3 and 7 dpi. **(C)** Quantitative analysis of phosphorylated Smad levels shown in B, depicted as ratio of ipsilateral versus contralateral levels for each time-point ($n = 3-5$ animals/group). **(D)** qRT-PCR analysis for *Bmp4*, *Bmp7*, *Tgfb1*, *Tgfb2* and *Tgfb3* mRNA levels at 3 and 7 dpi ($n = 5$ animals/group). **(E)** qRT-PCR analysis for *Noggin*,

Results

Both TGF β and BMP branches of the TGF β -superfamily signalling system are activated upon acute TBI within the wound area

To investigate the role of the TGF β -superfamily system and assess in parallel the activation of the TGF β and BMP branches upon brain injury, mice were subjected to unilateral SW injury of the right cerebral cortex, which represents a moderate model of TBI. One of the advantages of the model is that the injury is restricted only in the ipsilateral side, whereas the contralateral side of each animal can be used as control (Supplementary Fig. 1A). Tissues encompassing injured (ipsilateral) and corresponding intact (contralateral) areas were surgically micro-dissected (Fig. 1A) and hallmarks of TGF β -superfamily activation were analysed. Increased levels of phosphorylated (p) Smad1/5/8 were detected in protein extracts of the ipsilateral side at 1 and 3 days post-injury (dpi). Likewise, increased pSmad2 levels were observed at 1 dpi (Fig. 1B and C). Consistently, increased mRNA levels of ligands (*Bmp4*, *Bmp7*, *Tgfb1*, *Tgfb2* and *Tgfb3*) and known TGF β -superfamily target genes (*Id1*, *Smad6*, *Serpine1* and *Pmepa1*) were detected in the ipsilateral side at 3 and/or 7 dpi (Fig. 1D and E and Supplementary Fig. 1B). Interestingly, the trauma area was characterized by decreased mRNA and protein levels for Noggin, the natural BMP inhibitor (Fig. 1E and F), suggesting that BMP signalling upon TBI may be driven locally by altering the balance between ligands and corresponding inhibitors.

To further verify the activation of canonical BMP-mediated signalling and characterize potential cellular targets, available transgenic animals carrying BMP-responsive eGFP alleles (BRE-eGFP) were subjected to TBI. Injury led to substantial activation of the BMP reporter in cells with glial morphology around the trauma, which reached highest levels between 3 and 7 dpi (Fig. 2A and B). Interestingly, the area occupied by BRE-eGFP⁺ cells coincided with the area characterized by decreased Noggin immunoreactivity (Supplementary Fig. 2A). Increased expression of the astrocytic protein GFAP was found around the trauma already at 1 dpi and peaked at 7 dpi, when intense astrogliosis, as previously described (Mathewson and Berry, 1985), was observed throughout the ipsilateral cortex (Fig. 2A). The increased astrogliosis was verified by qRT-PCR and immunoblot analysis of

GFAP in dissected wound tissues (Supplementary Fig. 2B and C).

To characterize the BRE-eGFP⁺ cells, tissue sections from TBI-subjected animals were analysed by immunostaining with cell-lineage specific markers (Fig. 2C and D and Supplementary Fig. 3A). The vast majority of BRE-eGFP⁺ cells around the injury site co-expressed GFAP, ALDH1L1, GLUL and S100 β and thus were characterized as astrocytes (Fig. 2C and Supplementary Fig. 3A). Scattered BRE-eGFP⁺/GFAP⁺ cells were also detected across the ipsilateral cortex at 7 dpi (Supplementary Fig. 2D). Interestingly, none of the AIF1/IBA1⁺ microglia expressed the BRE-eGFP reporter, even when localized among numerous eGFP-expressing astrocytes (Fig. 2D).

Astrocytes and microglia exhibit differential responsiveness to TGF β 1 and BMP4 *in vitro*

To characterize the impact of TGF β or BMP signalling on activated glia cells and assess the failure to detect *in situ* activation of BRE-eGFP reporter in microglia, we analysed *in vitro* their responsiveness to TGF β 1 and BMP4, the two ligands exhibiting highest mRNA up-regulation within the injured tissues (Fig. 1D). Pure 'reactive' astrocytes and microglia were isolated by sorting mixed glia cultures prepared from neonatal mice, using anti-GLAST/SLC1A3 (astrocytes) and anti-CD11b/ITGAM (microglia) antibodies (Fig. 3A and Supplementary Fig. 3B). The purified cells, after a total of ~20–24 days in culture, were stimulated with recombinant ligands for 2 and 24 h, and their transcriptomes were analysed by RNA-Seq.

Upon TGF β 1 stimulation of purified astrocytes, 1069 genes were significantly upregulated and 513 downregulated at 2 h, whereas 1612 were upregulated and 1297 downregulated at 24 h. Similarly, upon BMP4 treatment, 1494 genes were upregulated and 719 downregulated at 2 h and 1702 genes were upregulated and 1568 downregulated at 24 h (Fig. 3B and C). Interestingly, TGF β 1- or BMP4-treated astrocytes exhibited highly overlapping transcriptional profiles 2 h post-stimulation, since ~47–69% of regulated genes responded to both ligands (Fig. 3B), albeit to different degrees (Supplementary Fig. 4A), whilst at 24 h, the gene expression profiles diverged exhibiting ~35–44% overlap (Fig. 3C). Few genes were oppositely regulated by these ligands (<2% and <8%, at 2 and 24 h, respectively), indicating limited antagonism between the two branches in astrocytes.

Id1, *Smad6*, *Serpine1* and *Pmepa1* mRNA levels, at 3 and 7 dpi ($n = 5$ animals/group). (F) Representative immunofluorescence images of coronal brain sections at the level of the wound area at 3 dpi stained for Noggin (white) and nuclei (DAPI, blue) (Scale bar = 150 μ m). Right image is a magnified caption (Scale bar = 30 μ m) of the area outlined with a magenta dotted square showing the glial morphology of Noggin-expressing cells. Data are expressed as mean \pm standard error of the mean (SEM) analysed using one-way analysis of variance with Bonferroni's *post hoc* test analysis (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). F values and degrees of freedom (df) are shown in parentheses for each data set.

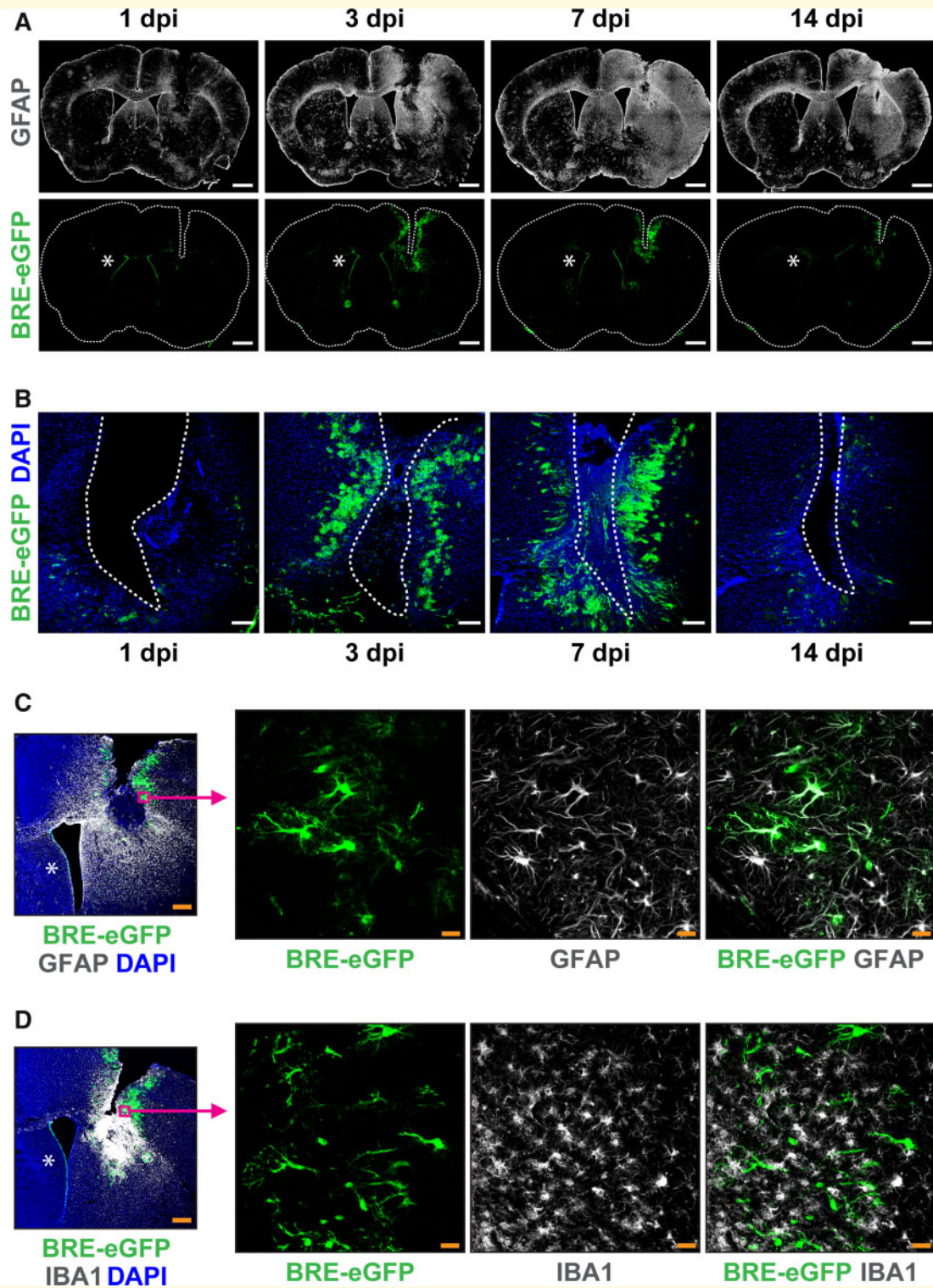


Figure 2 The BRE-eGFP reporter is activated upon acute TBI in astrocytes that demarcate the wound area. (A) Representative tile scan confocal immunofluorescence images of the astrocytic cytoskeletal protein GFAP (white, top row) and the BRE-eGFP reporter (green, bottom row) in coronal brain sections at the level of the wound at 1, 3, 7 and 14 dpi (Scale bar = 1mm). (B) Higher magnification immunofluorescence images of the ipsilateral side, showing the time-course of BRE-eGFP reporter expression (green) around the trauma (Scale bar = 150 μm). (C) Representative immunofluorescence image of the GFAP (white) and BRE-eGFP reporter (green) expression in the ipsilateral side at 7 dpi (left panel, Scale bar = 300μm). Magenta squares and arrows denote origin of higher magnification single layer images (right panel, Scale bar = 25μm) showing BRE-eGFP-positive cells co-expressing the GFAP protein. (D) Representative immunostaining for microglial marker AIFI/IBA1 (white) and BRE-eGFP (green) in the ipsilateral side at 7 dpi (left panel, Scale bar = 300 μm) and higher magnification single layer images (right panel, Scale bar = 25μm). DAPI (blue) is used for nuclear staining. Asterisks illustrate constitutive BRE-eGFP reporter expression in ependymal cells lining the medial walls of lateral ventricles.

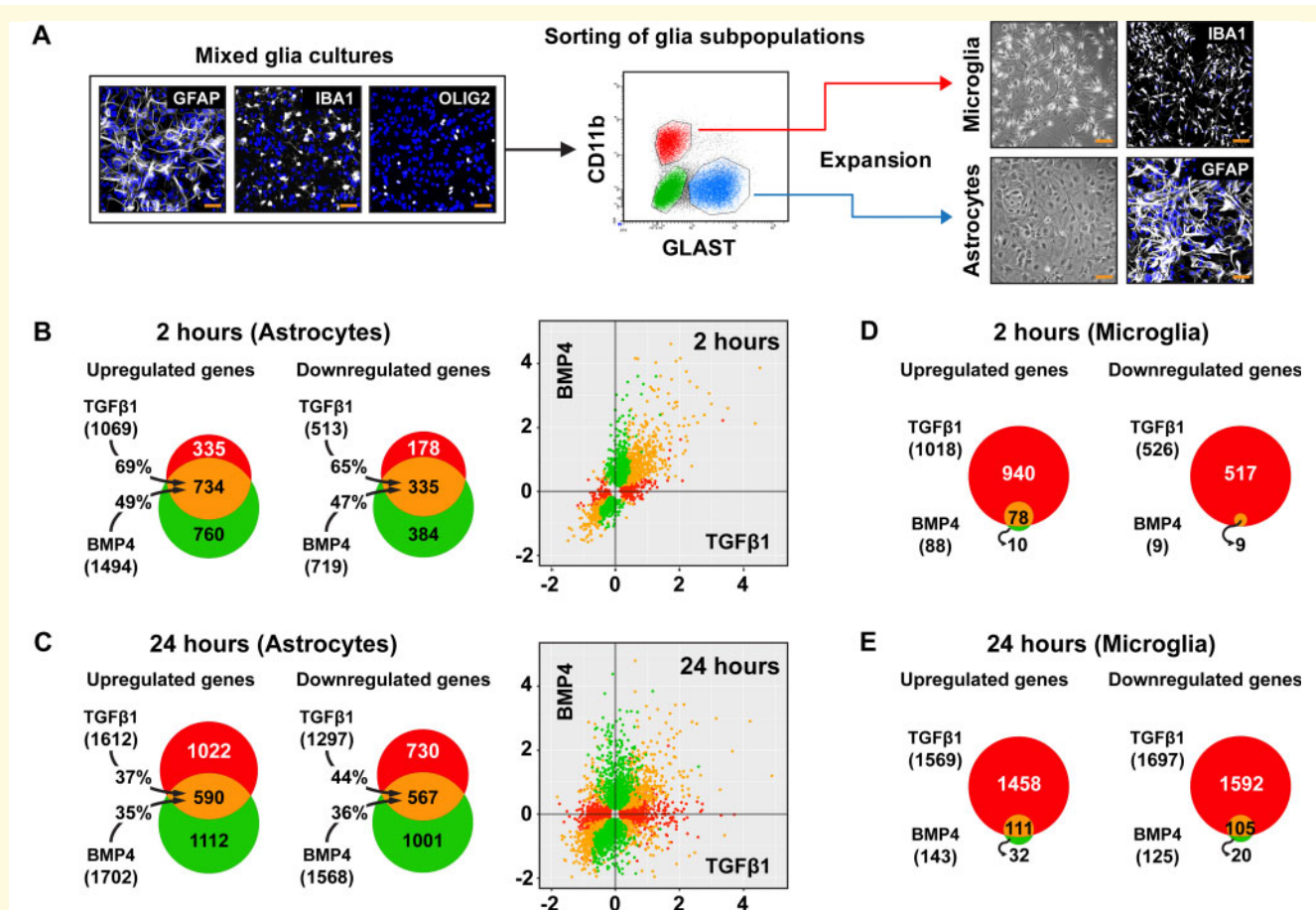


Figure 3 Purified astrocytes stimulated *in vitro* with TGFβ1 or BMP4 share highly overlapping transcriptomes, whereas, microglia respond selectively to TGFβ1. (A) Isolation of pure astrocytes and microglia from mixed glia cultures utilizing anti-GLAST (astrocytes) and anti-CD11b (microglia) antibodies. Mixed glia cultures, prepared from newborn mice, comprised of astrocytes (GFAP⁺), microglia (AIF1/IBA1⁺) and oligodendrocytes (OLIG2⁺); representative immunofluorescence image, left panel), were maintained for ~10–12 days prior to staining with antibodies against GLAST (blue gate) or CD11b proteins (red gate) for cell sorting. Purified astrocytes and microglia were expanded for additional ~10–12 days before analysis. Right panel shows representative bright field and immunofluorescence images of astrocyte and microglia cultures. DAPI (blue) is used for nuclear staining. Scale bar = 50 μm. (B and C) Venn diagrams summarizing RNA-Seq analysis results of purified astrocytes treated with 2 ng/ml TGFβ1 or 50 ng/ml BMP4 for 2 h (B) or 24 h (C) ($n = 3$ cultures/group). (D and E) Venn diagrams summarizing RNA-Seq analysis of purified microglia treated with 2 ng/ml TGFβ1 or 50 ng/ml BMP4 for 2 h (D) or 24 h (E) ($n = 3$ cultures/group). The diagrams depict numbers of statistically significant (baseMean > 30 and FDR < 0.05) up- or down-regulated genes by TGFβ1 (red), BMP4 (green) or both (orange). The percentages on the black arrows indicate the overlap between the two ligand treatments. The scatterplots in B and C show the genes with statistically significant response to TGFβ1 (red), BMP4 (green) or both (orange). The x- and y-axes represent log₂ fold change of differentially expressed genes.

Purified microglia responded remarkably well to TGFβ1, since 1018 genes were upregulated and 526 downregulated at 2h, whereas 1569 were upregulated and 1697 downregulated at 24h (Fig. 3D and E). Notably, astrocytes and microglia regulated distinct sets of genes in response to TGFβ1, exhibiting ~11–31% overlap (Fig. 4A). Surprisingly, still in agreement with the absence of BRE-eGFP reporter expression in AIF1/IBA1⁺ cells, purified microglia failed to respond robustly to BMP4. Only 88 genes were upregulated and 9 were downregulated at 2h. Similarly, 143 genes were upregulated and 125 were downregulated after 24h stimulation with BMP4. However, none exhibited log₂ fold change

>1.6, only 55 and 33 displayed log₂ fold change >0.4 or <-0.4, respectively, and the top upregulated genes included key negative regulators of the pathway, such as *Smad6*, *Smad7* and *Tgif1*.

To derive mechanistic explanation for the differential response of the two glial populations to TGFβ1 and BMP4, key components of the system were compared in astrocytes and microglia. Analysis of relative basal mRNA levels of TGFβ-superfamily system receptors and effector Smads demonstrated significant differences among them. Microglia were characterized by higher mRNA levels for *Acvr11/Alk1*, *Tgfr1/Alk5* and *Tgfr2*, compared with astrocytes that expressed higher mRNA levels for

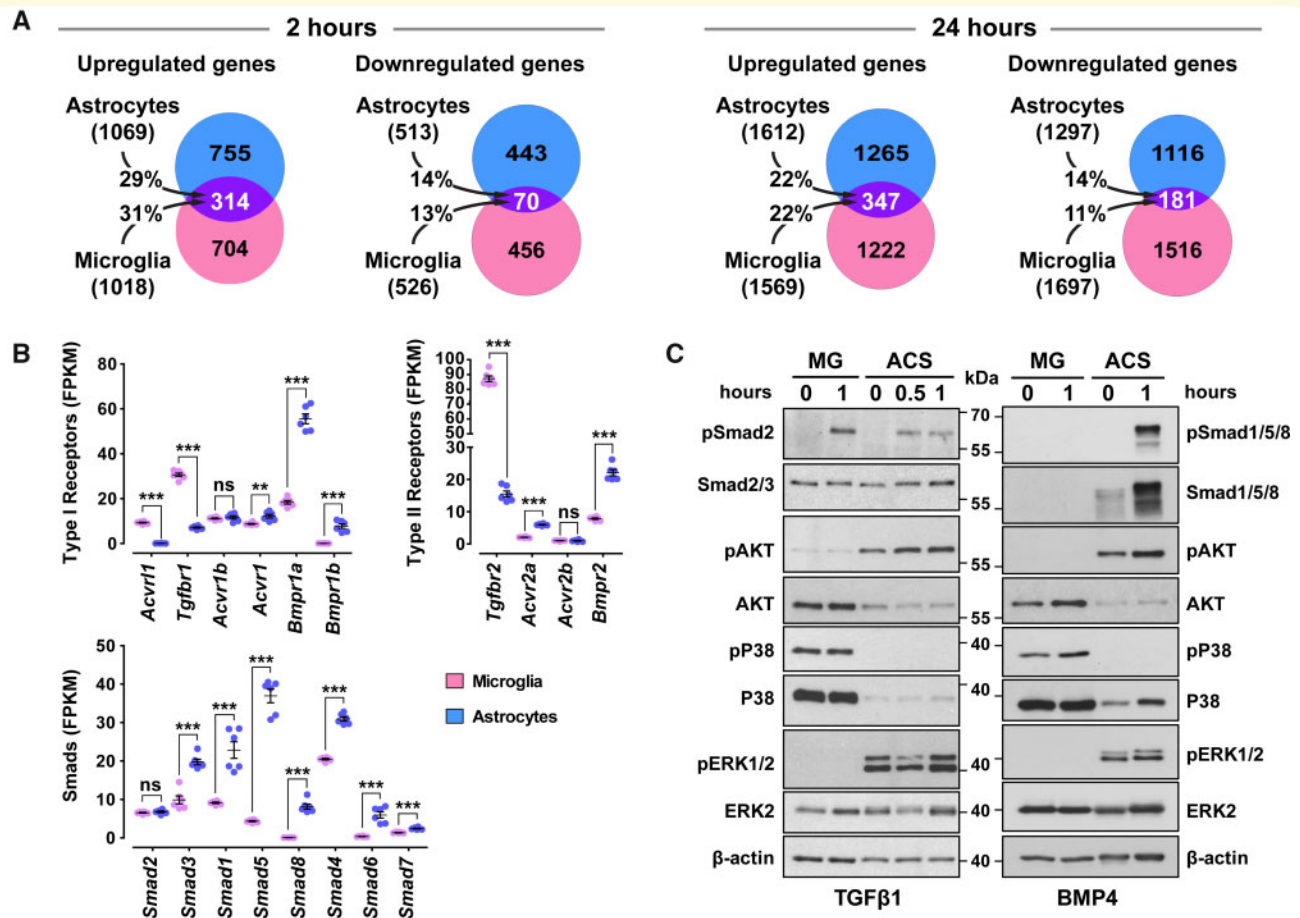


Figure 4 Astrocytes and microglia modulate expression of distinct sets of genes upon TGF β 1 treatment, express different components of the canonical pathways and are poised to activate different non-canonical pathways. **(A)** Venn diagrams representing the number of statistically significant (baseMean > 30 and FDR < 0.05) up- or down-regulated genes only in astrocytes (light blue), only in microglia (pink) or in both glial populations (purple), following stimulation with 2 ng/ml TGF β 1 for 2 h (left panel) or 24 h (right panel). The percentages indicate the gene overlap between astrocytes and microglia ($n = 3$ cultures/group). **(B)** mRNA expression levels of key components of the TGF β -superfamily system, including type-I receptors, type-II receptors and Smads, in vehicle-treated purified microglia (magenta dots) or astrocytes (blue dots) derived from RNA-Seq data that were normalized and expressed as Fragments Per Kilobase per Million reads mapped (FPKM) ($n = 6$ cultures/group). $^{***}P < 0.01$; $^{**}P < 0.001$; two-tailed unpaired t -test. **(C)** Representative immunoblot analysis of canonical and non-canonical TGF β -signalling pathways using antibodies against pSmad2, total Smad2/3, pSmad1/5/8, total Smad1/5/8, phosphorylated AKT (pAKT), total AKT, phosphorylated P38 (pP38), total P38, phosphorylated ERK1/2 (pERK1/2), total ERK2 and β -actin (loading control) in purified microglia (MG) and astrocytes (ACS) upon 10 ng/ml TGF β 1 (on the left) or 50 ng/ml BMP4 stimulation (on the right).

Acvr1/Alk2, *Bmpr1a/Alk3*, *Bmpr1b/Alk6*, *Acvr2a*, *Bmpr2* and *Smads* 1, 5, 8, 3, 4, 6 and 7 (Fig. 4B). Both cell types expressed similar mRNA levels for *Smad2*. Moreover, although *Smad2* was phosphorylated in both glial populations following TGF β 1 treatment, *Smad1/5/8* were phosphorylated upon BMP4 stimulation only in astrocytes (Fig. 4C). Consistently, total *Smad1/5/8* levels were below detection in the microglia population utilized in the analysis. Therefore, levels of BMP-related Smads and receptors may account for the low microglial response to BMP4. Interestingly, different non-canonical pathways were activated, even at baseline, in the two glial populations. Microglia were characterized by higher total and phosphorylated P38/MAP-kinase, whereas astrocytes exhibited higher phosphorylated ERK1/2 and

increased pAKT levels upon ligand stimulation (Fig. 4C), suggesting that TGF β -superfamily receptors could be wired with different non-canonical downstream signalling pathways in these two cell types.

Analysis of the transcriptomic profiles of TGF β 1- or BMP4-treated astrocytes using the IPA platform demonstrated that 2 h stimulation with either ligand led to activation of several canonical pathways related to growth factor-mediated signalling (TGF β , BMP, IGF1, Wnt/ β -catenin, ERK/MAPK, PI3K/AKT, STAT3, platelet-derived growth factor (PDGF), GDNF, neurotrophin/TRK, FGF, etc.), cytoskeletal remodelling and cell migration (ephrin receptor, integrin, actin cytoskeleton and glioma invasiveness signalling; Supplementary Fig. 5A). Twenty-four hours stimulation led to activation of pathways related to

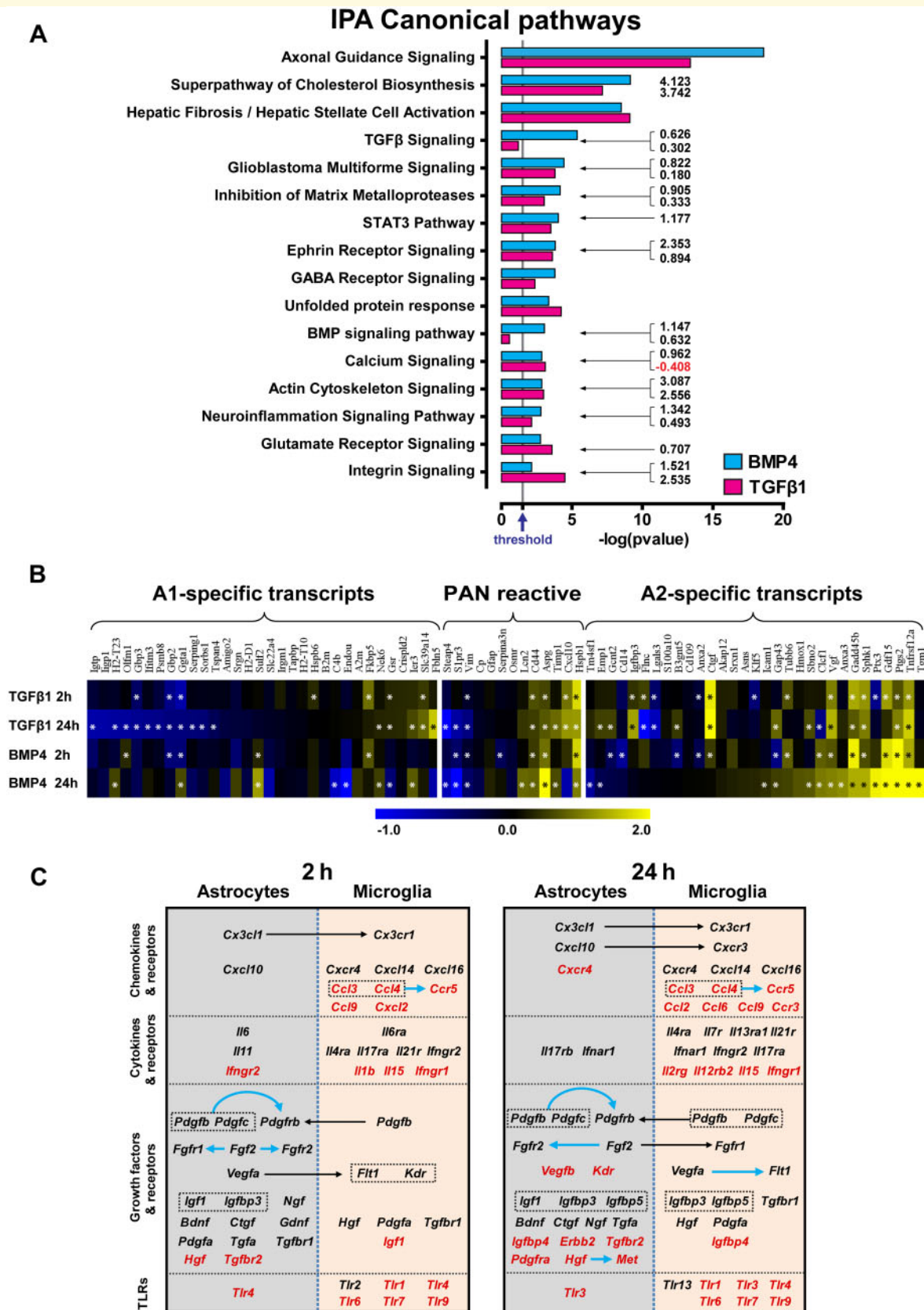


Figure 5 TGFβ1 or BMP4 stimulation guides purified astrocytes towards a reparatory, neuroprotective ‘A2’-like phenotype. **(A)** Graph depicting CNS-related IPA canonical pathways, selected among the top regulated, from RNA-Seq analysis of purified astrocytes stimulated with 2 ng/ml TGFβ1 or 50 ng/ml BMP4 for 24 h. The x-axis displays the statistical significance [-log (P value)] of the canonical pathways displayed along the y-axis. Grey vertical line, indicated by arrow in the x-axis, shows the threshold for statistical significance (P = 0.05). Numbers

tissue-repair/remodelling (Fig. 5A). The majority of identified Gene Ontology annotations (GOs) was regulated by both TGF β 1 and BMP4, with either distinct (Fig. 7C) or overlapping gene-sets modulated by either ligand (Figs 6A and B and 7A).

The apparent activation of tissue-repair and remodelling processes prompted us to investigate whether TGF β 1- or BMP4-stimulated astrocytes expressed genes associated with the 'A2'-like astrocytic phenotype described previously (Zamanian *et al.*, 2012; Liddelow *et al.*, 2017). As shown in Fig. 5B, both TGF β 1 and BMP4 shifted the profile of the astrocytes towards the 'A2'-like phenotype, and moreover, in agreement with Liddelow *et al.* (2017), TGF β 1 downregulated several genes associated with the 'A1'-like phenotype.

Analysis of TGF β 1-stimulated microglia demonstrated modulation of several pathways, some of which were also regulated in astrocytes, such as integrin-, actin cytoskeleton-, glioma invasiveness-, ephrin receptor- and PDGF-signalling (Supplementary Fig. 5B). However, various inflammation-related pathways were significantly modulated selectively in microglia. Those included, among others, production of nitric oxide, nuclear factor erythroid 2-related factor 2 (NRF2)-mediated oxidative stress response, vascular endothelial growth factor (VEGF) signalling, leucocyte extravasation, interferon and toll-like receptor signalling. The negative effect of TGF β -signalling on microglial inflammatory responses is well-documented (Spittau *et al.*, 2013; Taylor *et al.*, 2017; Zoller *et al.*, 2018). Consistently, already after 2 h stimulation, interferon and toll-like receptor signalling pathways were characterized by negative IPA z-scores (predicted decreased activity) and importantly, after 24 h the vast majority of modulated pathways was characterized by predicted decreased activity. Analysis of the expression of selected genes associated with the quiescent 'M0'-, pro-inflammatory 'M1'- and anti-inflammatory 'M2'-like microglia phenotypes demonstrated a downregulation of several 'M1'- and a shift towards an 'M0/M2'-like phenotype by TGF β 1 (Supplementary Fig. 5C).

Comparative analysis of TGF β 1-stimulated astrocytes and microglia revealed modulation of chemokines, cytokines, growth factors and corresponding receptors and pointed out potential TGF β -driven cross-interactions between the two populations (Fig. 5C). Specifically, *Cx3cl1*, *Cxcl10* and *Il6* mRNA levels were upregulated at 2 and/or 24 h in astrocytes and their corresponding receptors

were upregulated in microglia. *Pdgfs* and *Vegfa* were upregulated in astrocytes and microglia; however, *Pdgfrb* was selectively upregulated in astrocytes and *Flt1/Vegfr1* and *Kdr/Vegfr2* in microglia. *Fgf2* was upregulated in astrocytes and the corresponding receptors were upregulated at 2 h in astrocytes and at 24 h in both glial populations. The chemokines *Ccl3* and *Ccl4* and their receptor *Ccr5* were selectively downregulated in microglia. Interestingly, several toll-like receptors were downregulated in microglia or astrocytes. BMP4 regulated the above-mentioned astrocytic genes in a manner similar to TGF β 1, indicating that the two axes do not annul each other and may regulate the glia cross-talk cooperatively.

Since bioinformatics highlighted 'Axonal Guidance Signaling', 'Superpathway of Cholesterol Biosynthesis' and 'Hepatic Fibrosis/Hepatic Stellate Cell Activation' as the top pathways modulated in astrocytes after 24 h stimulation with both TGF β 1 and BMP4 (Fig. 5A), further analysis was focused on these injury-related processes.

Up-regulation of lipid metabolism components in astrocytes by TGF β -superfamily signalling or upon TBI

Remarkably, 20 out of the 24 enzymes involved in the 'Superpathway of Cholesterol Biosynthesis' (IPA) were upregulated in purified astrocytes upon 24 h treatment with TGF β 1 and/or BMP4, 13 of which were upregulated by both ligands (Fig. 6A). In addition, several components of lipid trafficking and metabolism, including transcription factors (*Srebf1*, *Srebf2*), transporters (*Abca1*, *Abcg4*), receptors (*Ldlr*, *Lrp1*), enzymes (*Lcat*, *Fasn*, *Acaca*) and other regulators (*Insig1*, *Insig2*, *Npc1*), were also upregulated by one or both ligands (Fig. 6B). Interestingly, few genes within this pathway (*Sqle*, *Abca1*, *Abcg4*, *Lcat*) were oppositely regulated by the two ligands (Fig. 6A and B). The response of selected genes encoding for cholesterol biosynthesis enzymes (*Hmgcr*, *Cyp51*, *Idi1*, *Dhcr24*, *Sqle*, *Sc5d*) or lipid metabolism components (*Srebf1*, *Srebf2*, *Abca1*, *Abcg4*, *Ldlr*, *Lcat*) was verified by qRT-PCRs of independent ligand-stimulated cultures (Supplementary Fig. 6A and B). In agreement with the *in vitro* stimulated cultures, mRNA levels for *Srebf1* and *Abca1* in the TBI-subjected animals were increased at 3 and 7 dpi and *Abcg4* levels were decreased

at the right side of the bars represent calculated IPA z-scores predicting an increased (black numbers) or a decreased (red numbers) pathway activity. (B) Heat map depicting log₂ fold changes of differentially expressed PAN reactive, 'A1'- and 'A2'-specific reactive astrocyte transcripts (according to Zamanian *et al.*, 2012 and Liddelow *et al.*, 2017), as determined by RNA-Seq analysis of purified astrocytes following 2 ng/ml TGF β 1 or 50 ng/ml BMP4 stimulation for 2 or 24 h (baseMean > 30). Asterisks depict statistical significance (FDR < 0.05). (C) Schematic representation of the expression of chemokines, cytokines, growth factors and relevant receptors, derived from RNA-Seq analysis of purified microglia and astrocytes treated with 2 ng/ml TGF β 1 for 2 h (left panel) or 24 h (right panel). Upregulated genes are shown in black, whereas the downregulated are shown in red. Black and blue arrows depict potential heterotypic or homotypic interactions, respectively, between glial populations.

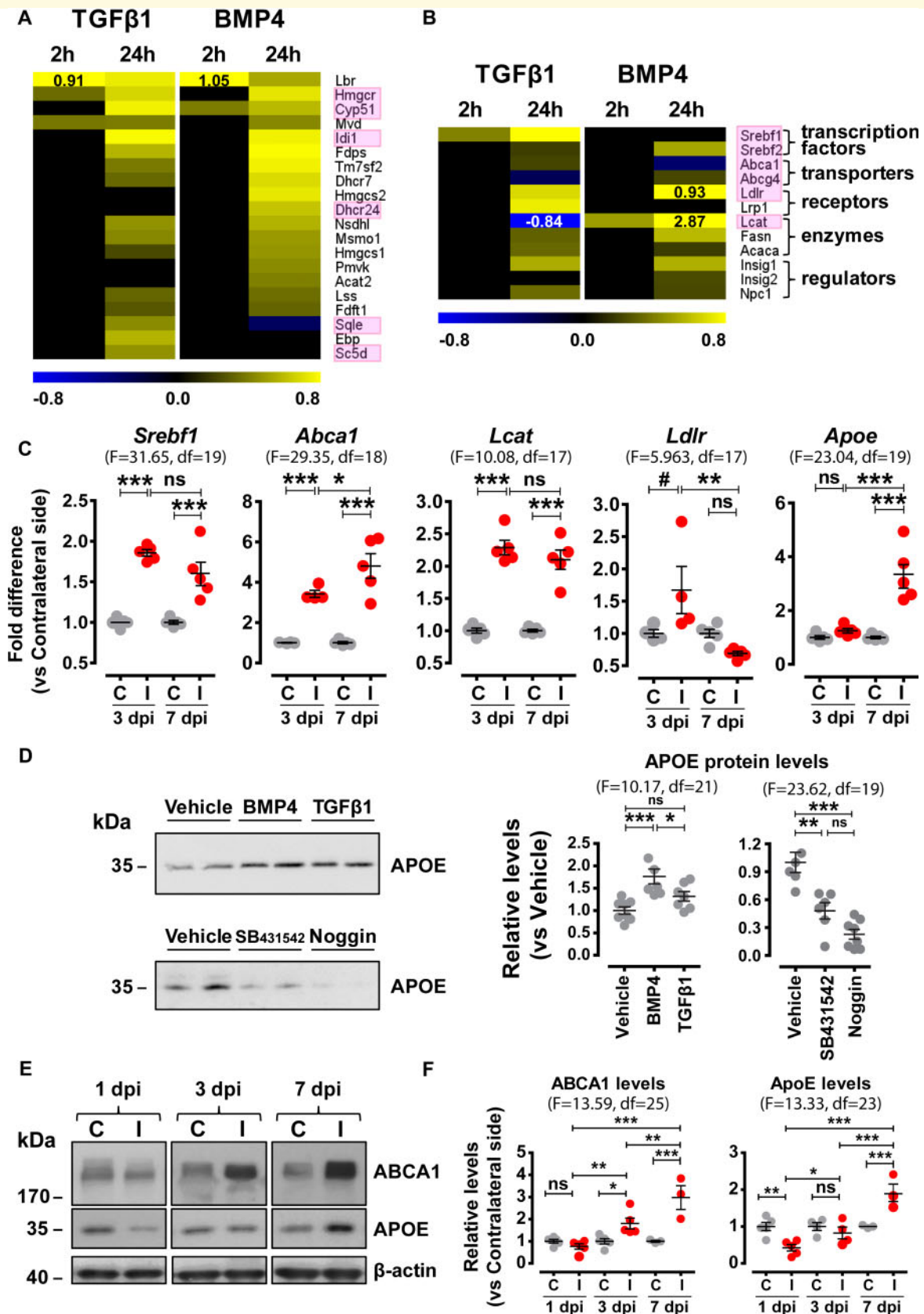


Figure 6 Lipid biosynthesis, metabolism and trafficking components are upregulated upon TBI in the wound area and in purified astrocytes upon *in vitro* stimulation with TGFβ1 or BMP4. Heat maps depicting log₂ fold changes of differentially expressed genes encoding (A) cholesterol biosynthesis enzymes or (B) lipid trafficking and metabolism components, as determined by RNA-Seq analysis of purified astrocytes following 2 ng/ml TGFβ1 or 50 ng/ml BMP4 stimulation for 2 or 24 h (baseMean > 30 and FDR < 0.05). Black or white

at 3 dpi in the ipsilateral side (Fig. 6C). Similarly, mRNA expression of *Lcat* was increased at the wound area at 3 and/or 7 dpi.

Since astrocytes are the main cholesterol producers and apolipoprotein-E (APOE) is the principal cholesterol carrier in adult brain, further analysis was focused on the production and secretion of APOE upon TGF β 1 or BMP4 stimulation. Increased levels of secreted APOE were detected in supernatants of astrocyte cultures treated with ligands for 24 h (Fig. 6D). Consistently, decreased APOE levels were found in the supernatants of astrocytes treated with either the TGF β -receptor kinase inhibitor SB431542 or the BMP-inhibitor Noggin. Moreover, *ApoE* mRNA expression was increased in the ipsilateral side following SW at 7 dpi (Fig. 6C). At protein level, APOE was significantly decreased at 1 dpi, reached contralateral side levels at 3 dpi and then increased at 7 dpi (Fig. 6E and F). ABCA1 protein levels were also increased in the ipsilateral side, at 3 and 7 dpi.

TGF β -superfamily system modulates processes related to axonal guidance and tissue-repair

Expression of several astrocyte-related axonal guidance molecules (Anderson *et al.*, 2016) was modulated upon treatment of astrocytes with either TGF β 1 or BMP4 (Fig. 7A). Interestingly, TGF β 1 induced a more axonal-permissive profile, whereas BMP4 upregulated equal numbers of inhibitory and permissive molecules. Expression of representative inhibitory and permissive molecules was analysed in tissues from TBI-subjected animals. In line with the *in vitro* findings, *Ncan*, *Nrp*, and notably, *Tnc* and *Lif*, two transcripts reportedly highly upregulated in ‘A2’-astrocytes (Zamanian *et al.*, 2012), were upregulated in the ipsilateral side at 3 dpi (Fig. 7B).

Numerous genes in the ‘Hepatic Fibrosis’ pathway (IPA), including collagens, growth factors, growth factor receptors and other tissue-remodelling components were modulated in astrocytes following TGF β 1 or BMP4 stimulation (Fig. 7C and Supplementary Fig. 7A). Interestingly, marginally overlapping sets of collagens were regulated by the two ligands. The majority of genes encoding growth factors and receptors was regulated early at 2 h, in contrast with other molecules in this annotation that were regulated after

24 h treatment. Representative genes upregulated in ligand-treated astrocytes were also found upregulated within the injured tissue, following TBI. Specifically, *Col1a1*, *Col5a2*, *Il6* and *Mmp2* were upregulated in the ipsilateral side at 3 and 7 dpi (Fig. 7D and Supplementary Fig. 7B). Notably, increased Collagen-I deposition was detected in the vasculature of injured areas and processes of BRE-eGFP⁺ astrocytes consistently were found closely interacting with vessel walls characterized by intense Collagen-I immunoreactivity (Fig. 7E).

Evidently, some genes belonging to the GO annotations analysed above responded differently in the *in vitro* stimulated astrocytes and the ipsilateral regions dissected from SW-subjected animals. Specifically, mRNA levels for *Sreb2* (‘Superpathway of Cholesterol Biosynthesis’, IPA) were upregulated *in vitro* by TGF β 1 and/or BMP4 but were downregulated within the ipsilateral tissue at 3 and/or 7 dpi (Supplementary Fig. 6C). Likewise levels of *Hmgcr* and *Cyp51* were not found altered significantly in the dissected TBI regions. Despite the changes in *ApoE* mRNA levels in the dissected tissues, this gene was not affected by ligand stimulation *in vitro*. Also, the *in vitro* observed modulation of *Ctgf/Ccn2* and *Igf1* (‘Hepatic Fibrosis’, IPA) could not be reproduced in acute TBI (Supplementary Fig. 7B). These differences could reflect inability of *in vitro* cultures of purified cells to simulate completely the complex cellular interactions that occur within the injured tissues, the possible differences in the kinetics by which certain processes unfold *in vitro* versus *in vivo*, or the assay’s detection limitations. Nevertheless, overall, the *in vitro* RNA-Seq data were in good agreement with the *in vivo* expression patterns revealed through the analysis of dissected trauma tissues.

Discussion

To assess the involvement of both TGF β and BMP branches of the TGF β -superfamily signalling system in TBI pathophysiology, we utilized a SW brain injury model and analysed the expression of key molecules within the injured tissues. We supplemented our *in vivo* study with gene expression profiling of TGF β 1- or BMP4-stimulated purified ‘reactive’ astrocytes and microglia. Our study demonstrated that upon brain injury both

numerical values indicate log₂ fold differences exceeding the colour scale bar limits. Pink frames indicate selected genes whose expression was verified with independent qRT-PCR analysis (Supplementary Fig. 6A and B). (C) qRT-PCR analysis of mRNA expression of *Sreb1*, *Abca1*, *Lcat*, *Ldlr* and *ApoE* in dissected contralateral (C) or ipsilateral (I) regions of TBI-subjected animals at 3 and 7 dpi (n = 5 animals/group). (D) Representative immunoblots and quantitative analysis of secreted APOE protein levels in cultures of purified astrocytes treated with 2 ng/ml TGF β 1, 50 ng/ml BMP4, 100 ng/ml Noggin or 10 μ M SB431542 for 24 h (n = 6–8 cultures/group from three independent experiments). (E) Representative immunoblots of ABCA1, APOE and β -actin (loading control) in the contralateral (C) and ipsilateral (I) dissected tissues of TBI-subjected animals at 1, 3 and 7 dpi (n = 3–5 animals/group). (F) Quantitative analysis of ABCA1 and APOE protein levels shown in E, depicted as ratio of ipsilateral versus contralateral levels for each time-point. Data are expressed as mean \pm standard error of the mean (SEM) analysed using one-way analysis of variance with Bonferroni’s *post hoc* test analysis (**P* < 0.05, ***P* < 0.01, ****P* < 0.001) or Newman–Keuls Multiple Comparison Test (#*P* < 0.05). *F* values and degrees of freedom (df) are shown in parentheses for each data set.

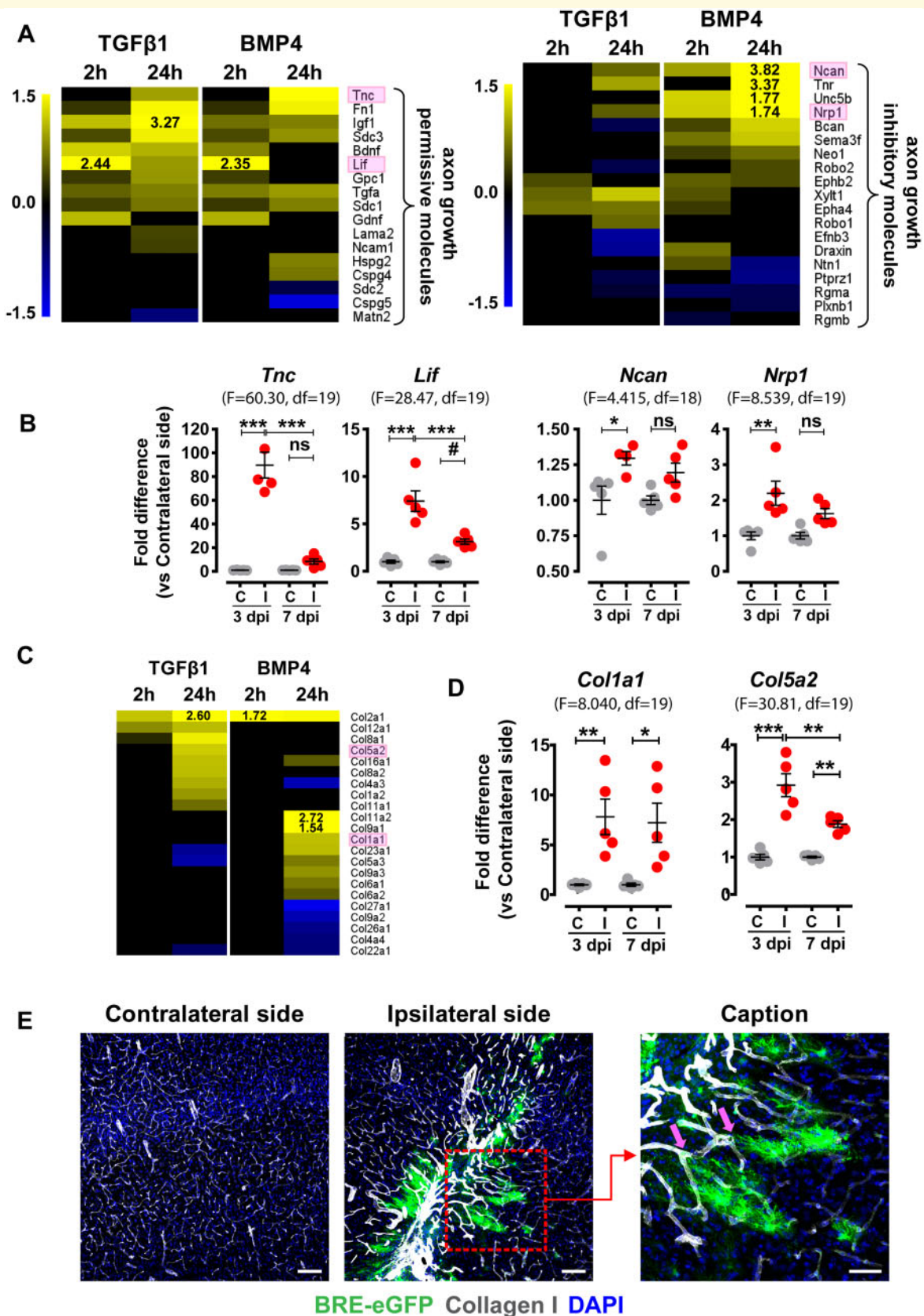


Figure 7 Genes encoding for axonal guidance and tissue-repair components are modulated *in vitro*, upon stimulation of purified astrocytes with TGFβ1 or BMP4, or *in vivo*, upon acute TBI. (A and C) Heat maps depicting log₂ fold changes of differentially expressed genes encoding (A) axonal guidance molecules [with permissive or inhibitory action, as summarized in Anderson et al. (2016)] and (C) collagens ('Hepatic Fibrosis' pathway, IPA), derived from RNA-Seq analysis of purified astrocytes stimulated with 2 ng/ml TGFβ1 or 50 ng/ml

TGF β and BMP axes are activated, as exemplified by increased levels of TGF β and BMP pSmads, and mRNA for relevant ligands and target genes in the lesion area during the first dpi (Fig. 1). Activation of the TGF β axis in astrocytes has already been associated with TBI (Schachtrup *et al.*, 2010); however, this is the first time that activation of the BMP axis is clearly demonstrated within the injured area. Utilizing the BRE-eGFP reporter mice, we showed that activation of canonical BMP pathway was confined to astrocytes demarcating the transition between healthy and damaged tissue, a pattern reminiscent of the increased pSmad1/5/8 levels in GFAP⁺ astrocytes at the injury site in a rat model of demyelinating spinal cord injury (Fuller *et al.*, 2007). Although substantial up-regulation of *Tgfb1* mRNA levels was demonstrated in TBI tissues, corresponding changes in BMP-encoding transcripts were not as robust (Fig. 1D). Instead, TBI areas were characterized by reduced Noggin mRNA and protein levels (Fig. 1E, F and Supplementary Fig. 2A), suggesting that the trauma core favours activation of the BMP axis primarily through the establishment of an inhibitor-deficient microenvironment.

Up-regulation of BMP2/4, BMP7 and Noggin (with the latter being expressed almost exclusively by reactive astrocytes at the injury site) in penetrating brain and spinal cord injuries has been previously reported (Hampton *et al.*, 2007). On the other hand, in an experimental model of stroke, Noggin expression mainly in activated microglia began to increase after 2 weeks and was further increased at 4 weeks only in the ischaemic subcortex, but the intensity was weak compared with the intensity of BMPs (Shin *et al.*, 2012). Earlier studies with transgenic animals overexpressing constitutively Noggin in neurons provided evidence in favour of a protective role of Noggin upon ischaemia-induced brain injury (Samanta *et al.*, 2010). However, these studies cannot be compared directly to the current study because Noggin overexpression was forced experimentally through the entire life of the animals leading most likely to readjustments of their homeostatic set points, as exemplified by the higher density of microglia in the tissues of non-manipulated animals (Samanta *et al.*, 2010). Moreover, conditional deletion of the BMP type-II receptor (BMPRII) in NG2-expressing cells after hypoxic-ischaemic injury globally protected the brain and prevented loss of

oligodendroglia (Dettman *et al.*, 2018). Thus, Noggin may have distinct roles in the processes of glial scar formation and neuro-restoration depending on the model utilized, the mode and the timing of intervention.

The kinetics of BRE-eGFP reporter and pSmad activation suggested that the canonical TGF β -superfamily system is not active in astrocytes immediately after injury but rather at later stages when these cells are already activated within the injured tissues. Therefore, insights regarding the way by which TGF β s and BMPs could modify the functionality of already activated astrocytes *in vivo* were obtained, indirectly, by treating with TGF β 1 or BMP4 purified reactive astrocytes that were derived from neonatal mixed glia cultures grown *in vitro* for ~20–24 days. Earlier studies (Cahoy *et al.*, 2008; Foo *et al.*, 2011; Zamanian *et al.*, 2012) have shown that astrocytes derived from such cultures share many of the characteristics of adult reactive astrocytes and in hierarchical clustering analysis they cluster closer to ‘A2’-like reactive astrocytes and away from both quiescent and LPS-reactive, ‘A1’-like, astrocytes (Zamanian *et al.*, 2012). Interestingly, they express ~60% of the ‘A2’-like and ~50% of the ‘A1’-like phenotype characteristic marker genes (Zamanian *et al.*, 2012), representing thus a suitable population to study factors that can modify the astrocytic ‘A1’/‘A2’-phenotypic plasticity. Indeed, both TGF β 1 and BMP4 shifted the profile of cultured astrocytes towards the ‘A2’ phenotype, and moreover, in agreement with Liddel *et al.* (2017), TGF β 1 downregulated several genes associated with the ‘A1’ phenotype, suggesting that both TGF β and BMP signalling systems could be important regulators of reactive astrocyte plasticity.

Transcriptomic analysis provided clues regarding the interplay between TGF β and BMP axes and the responses they could potentially evoke *in vivo*. Interestingly, activation of astrocytes with either ligand affected highly overlapping sets of genes. Up to 69% of these genes responded to both TGF β 1 and BMP4 after 2 h stimulation (Fig. 3B), modulating pathways related to growth factor-mediated signalling, cytoskeleton remodelling and cell migration (Supplementary Fig. 5A). Twenty-four hours post-stimulation, when primarily processes related to tissue-repair and remodelling were modulated, the

BMP4 for 2 or 24 h (baseMean > 30 and FDR < 0.05). (B) qRT-PCR analysis of mRNA expression of axonal growth permissive molecules *Tnc* and *Lif* and axonal growth inhibitory molecules *Ncan* and *Nrp1* (highlighted with pink frames in A) in the contralateral (C) and ipsilateral (I) dissected tissues from TBI-subjected animals at 3 and 7 dpi ($n = 5$ animals/group). (D) qRT-PCR analysis of *Coll1a1* and *Col5a2* (highlighted with pink frames in C) mRNA expression in the contralateral (C) and ipsilateral (I) tissues dissected from TBI-subjected animals at 3 and 7 dpi. Data are expressed as mean \pm standard error of the mean (SEM) analysed using one-way analysis of variance with Bonferroni's *post hoc* test analysis (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) or Newman–Keuls Multiple Comparison Test (# $P < 0.05$). *F* values and degrees of freedom (df) are shown in parentheses for each data set. (E) Representative immunofluorescence images for Collagen-I (white) and BRE-eGFP (green) expression in coronal brain sections at the level of the wound at 7 dpi (Scale bar = 100 μ m). Red dashed square indicates area shown in higher magnification (Scale bar = 50 μ m) of BRE-eGFP-expressing astrocytes interacting with vasculature characterized by intense Collagen-I deposition (pink arrows). DAPI (blue) is used for nuclear tracing.

TGF β 1 and BMP4 transcriptomes diverged; however, the overall overlap was still up to \sim 44% (Figs 3C and 5A).

The substantial overlap between TGF β 1- and BMP4-induced genes in astrocytes, especially at the early stage of treatment, may be explained by the reported property of TGF β to activate BMP-Smads, through an interplay of TGFBR1/ALK5 with either ACVRL1/ALK1 (Goumans et al., 2002) or ACVR1/ALK2 (Ramachandran et al., 2018). Consistently, astrocytes express mRNA for *Acvr1/Alk2* (Fig. 4B) and transient increase in pSmad1/5/8 levels was always observed in TGF β 1-treated astrocytes (Supplementary Fig. 4B). These findings support the notion that transcriptional responses evoked by TGF β -superfamily require integrated combinatorial signalling via both Smad2/3 and Smad1/5/8 pathways (Ramachandran et al., 2018).

Despite the aforementioned overlap, the sets of TGF β 1- or BMP4-modulated genes within each GO annotation differed qualitatively and quantitatively, indicating that each ligand could stimulate qualitatively different functional outcomes. Firstly, commonly regulated genes responded to each ligand to a different degree. Hence, classical TGF β targets such as *Serpine1* responded more robustly to TGF β 1, whereas typical BMP targets such as *Id1*, *Smad6* and *Noggin* responded more robustly to BMP4 stimulation (Supplementary Fig. 4A). Secondly, within each commonly regulated pathway, some genes responded to both ligands, and some were modulated selectively by either TGF β 1 (*Ctgf/Ccn2*, *Col12a1*) or BMP4 (*Gata2*, *Bambi*). The ratio of commonly and selectively modulated genes differed among pathways.

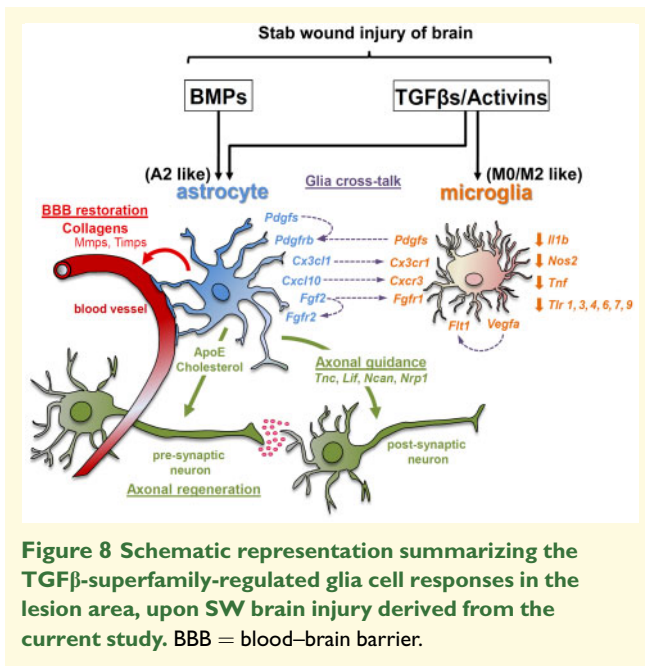
To investigate the effect of the TGF β -superfamily signalling system on microglia, we utilized CD11b⁺GLAST⁻ cells derived from 14 days old mixed glia cultures of neonatal brains that were cultured as pure microglia for additional 10–12 days (\sim 24 days in total). Interestingly, such cultured microglia responded remarkably well to TGF β 1, however, consistently with the failure to detect *in vivo* activation of the BRE-eGFP reporter in adult microglia upon TBI (Fig. 2D), failed to respond robustly to BMP4 and expressed relatively lower mRNA levels for BMP receptors and (Fig. 4B), suggesting that microglia might be programmed to be insensitive to BMP signalling.

It is currently recognized that neonatal microglia exhibit an ‘immature’ phenotype and do not express genes that characterize adult microglia (Butovsky et al., 2014). However, the cells utilized herein, probably due to their prolonged *in vitro* growth in the presence of serum, although they retained some characteristics of ‘immature’ microglia, such as high mRNA levels for *ApoE*, yet, they expressed half of the genes that constitute the ‘adult microglial’ molecular signature (40/81 genes with FPKM from 20 to 7500) and several genes that have been associated with the reportedly (Butovsky et al., 2014) ‘M0’, ‘M1’- and ‘M2’-like phenotypes (15/38 ‘M0’, 21/52 ‘M1’ and 24/46 ‘M2’ marker genes). Moreover, 42 out of 54 genes that have been found to constitute a TGF β

dependent signature of adult microglia were also found herein modulated by TGF β 1 in the *in vitro* microglia cultures (32 upregulated and 10 downregulated; Supplementary Fig. 5C), suggesting that these cells may provide a useful model for studying the effect of the TGF β -superfamily signalling system on microglial molecular plasticity. Indeed, TGF β 1 upregulated 22 out of 35 known ‘M0’, upregulated 25 out of 44 known ‘M2’ and downregulated 30 out of 44 known ‘M1’ marker genes, suggesting that TGF β can shift the molecular profile of activated microglia away from the pro-inflammatory ‘M1’ phenotype and towards the quiescent or anti-inflammatory ‘M0/M2’ phenotypes, respectively.

TGF β 1 modulated expression of several chemokines, cytokines, growth factors and receptors in astrocytes and microglia. Up-regulation of a ligand in one cell-type and its cognate receptor(s) on the other, as exemplified by the *Pdgfs/Pdgfrb*, *Cx3cl1/Cx3cr1* and *Cxcl10/Cxcr3* pairs (Figs 5C and 8) might highlight potential TGF β -driven cross-interactions between them. Notably, the CX3CL1/CX3CR1 axis has been associated with reduced neurotoxicity and microglial activation (Mizuno et al., 2003; Pabon et al., 2011; Febinger et al., 2015). Likewise, PDGFs have been associated with survival signals upon CNS injury (Funa and Sasahara, 2014) and the CXCL10/CXCR3 axis has been linked to microglial recruitment to the injury site and subsequent neuronal re-organization (Rappert et al., 2004; Li et al., 2006). Moreover, pairs of ligands and cognate receptors were modulated in the same population, specifically, *Fgf2/Fgfr1:Fgfr2* and *Pdgfb:Pdgfc/Pdgfrb* were upregulated in astrocytes, *Vegfa/Flt1:Kdr* were upregulated and *Ccl3:Ccl4/Ccr5* downregulated in microglia, highlighting thus potential homotypic interactions. Notably, activation of the FGF pathway has been associated with suppression of the ‘A1’ astrocytic phenotype (Kang et al., 2014; Liddel et al., 2017). Hence, it appears that another way by which TGF β 1 could indirectly modulate microglial reactivity is by up-regulating anti-inflammatory mediators in reactive astrocytes and their receptors in microglia.

Maximal activation of BRE-eGFP reporter occurred between 3 and 7 dpi, a period associated with initiation of tissue-repair. Therefore, particular emphasis was given to the effect of TGF β 1 or BMP4 on astrocytes at 24 h, when the top canonical pathways modulated by both ligands were the ‘Superpathway of Cholesterol Biosynthesis’, ‘Axonal Guidance Signaling’ and ‘Hepatic Fibrosis’. In adult brain, astrocytes are the major cholesterol providers (Nieweg et al., 2009) and their lipid metabolism is critical for synapse development and function (van Deijk et al., 2017). Interestingly, almost all enzymes involved in the superpathway of cholesterol biosynthesis together with various components of lipid transport and metabolism were upregulated in ligand-treated astrocytes (Fig. 6A and B). Activation of this pathway upon TBI was verified by demonstrating up-regulation of several components, such as *Srebf1*, *Abca1*, *Ldlr* and the



extracellular cholesterol-esterifying enzyme *Lcat*, within the injured tissues (Fig. 6C). The increased *Lcat* expression could relate to the significant increase of esterified cholesterol following cortical impact injury (Roux *et al.*, 2016). Moreover, secreted APOE was increased upon BMP4 or TGF β 1 stimulation of astrocytes *in vitro*, and decreased when inhibitors of TGF β -signalling system were applied (Fig. 6D). Consistently, APOE and ABCA1 were gradually upregulated both at mRNA and protein levels, within the SW-injured tissues (Fig. 6C, E, and F). Interestingly, APOE protein levels were significantly reduced at 1 dpi, normalized at 3 dpi and increased at 7 dpi. Pro-inflammatory cytokines such as TNF α , IL1 β and IFN γ suppress APOE production (Zhang *et al.*, 2011). It is possible that an early burst of such cytokines (at 1 dpi) in the wound microenvironment could account for the observed decreased APOE protein levels at this early time-point. The anti-inflammatory and APOE-inducing function of the TGF β -superfamily signalling that is mobilized after 3 dpi could be responsible for the late increase in both mRNA and protein levels of APOE in the injured tissues. This hypothetical scenario warrants further investigation. Collectively, our findings implicate for the first time TGF β and BMP signalling in the regulation of cholesterol biosynthesis, APOE secretion and possibly lipoprotein formation following interaction of APOE with ABCA1 cholesterol transporter and LCAT esterifying enzyme in astrocytes.

TGF β 1 and BMP4 regulated several molecules with axonal guidance properties. Interestingly, TGF β 1 upregulated primarily axonal-permissive molecules and downregulated inhibitory ones, whereas BMP4 upregulated both permissive and inhibitory genes (Fig. 7A). Increased expression of both permissive and inhibitory axonal growth

molecules was verified within the injured tissues (Fig. 7B) suggesting that astrocytes demarcating the wound could provide directionally the signals required for axonal regeneration (Jones *et al.*, 2003; Faulkner *et al.*, 2004; Cregg *et al.*, 2014). Congruently, earlier studies have shown that BMP4 up-regulates neurocan (*Ncan*) and brevican (*Bcan*), and down-regulates phosphacan (*Ptprz1*) mRNA in spinal cord astrocytes (Fuller *et al.*, 2007) and TGF β 1 increases mRNA and protein levels of neurocan in cortical astrocytes (Asher *et al.*, 2000; Schachtrup *et al.*, 2010).

Plethora of tissue-remodelling components, including collagens, growth factors and receptors were upregulated in astrocytes by TGF β 1 or BMP4. Interestingly, the two ligands regulated different collagens, supporting the notion that TGF β and/or BMP signalling may play distinct roles during tissue-repair. Up-regulation of selected genes, regulated *in vitro* selectively by TGF β 1 (*Col5a2*, *Mmp2*), BMP4 (*Col1a1*) or both (*Il6*), was verified by analysing dissected wound regions (Fig. 7C and D and Supplementary Fig. 7B). Notably, the visualization of processes of BRE-eGFP⁺ astrocytes in close association to vessel walls with increased Collagen-I immunoreactivity (Fig. 7E), strongly suggests that BMP-activated astrocytes could play a key role in sealing disrupted vasculature and restoring blood–brain barrier.

TBI represents one of the principal causes of death and disability worldwide and ~50% of the world's population will have at least one TBI incidence over their lifetime (Maas *et al.*, 2017; Quaglio *et al.*, 2017). Despite the rapidly increasing body of literature, TBIs remain especially challenging to treat due to their heterogeneous nature and the induction of complex pathogenesis pathways that, still, are not fully understood (Saatman *et al.*, 2008). Therefore, clarification of the molecular processes underlying TBI pathophysiology, in particular, the cross-talk between astrocytes and microglia and the mechanisms that modulate plasticity of activated glial cells will be important steps towards the development of therapeutic strategies that could harness their reparatory capacity (Sofroniew, 2009).

Our study provides a comprehensive analysis of the potential role of both branches of the TGF β -superfamily signalling system in the context of acute focal TBI pathology and draws attention on the capacity of both TGF β and BMP signalling to regulate key processes for tissue-injury repair and glial pro-inflammatory activity, in activated astrocytes and microglia surrounding the trauma (Fig. 8). Both TGF β and BMP act on astrocytes and induce highly overlapping transcriptional programmes, whereas microglia respond selectively to TGF β signalling. Thus, selective manipulation of either branch of the TGF β -superfamily, especially in astrocytes, should be treated with caution given the potential compensatory activation or inhibition of the other. The SW injury utilized herein represents probably the simplest form of TBI that does not simulate the biomechanical aspects of human TBI. However, the wealth of information obtained

herein could be further utilized to study more complex models that simulate closer human TBI pathophysiology, ideally with genetically modified animals and inducible ablation of specific components of the TGF β -superfamily signalling system (receptors, ligands or inhibitors) specifically in astrocytes or microglia and thus aid prospectively to the design of therapeutic strategies for TBI and similar neuro-inflammatory conditions.

Supplementary material

Supplementary material is available at *Brain Communications* online.

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Competing interests

The authors report no competing interests.

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