Disorders of the Nervous System

TGFβ1 Induces Axonal Outgrowth via ALK5/PKA/SMURF1-Mediated Degradation of RhoA and Stabilization of PAR6

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Visual Abstract

Transforming growth factor (TGF)β1 has repeatedly been associated with axonal regeneration and recovery after injury to the CNS. We found TGFβ1 upregulated in the stroke-denervated mouse spinal cord after ischemic injury to the motor cortex as early as 4 d postinjury (dpi) and persisting up to 28 dpi. Given the potential role of TGFβ1 in structural plasticity and functional recovery after stroke highlighted in several published studies, we investigated its downstream signaling in an in vitro model of neurite outgrowth. We found that in this model, TGFβ1 rescues neurite outgrowth under growth inhibitory conditions via the canonical TGFβR2/ALK5 signaling axis. Thereby, protein kinase A (PKA)-mediated phosphorylation of the E3 ubiquitin ligase SMURF1 induces a switch of its substrate preference from PAR6 to the Ras homolog A (RhoA), in this way enhancing

Significance Statement

This study addresses a mechanism for Transforming growth factor (TGF)β1 to increase compensatory axonal sprouting and growth after cortical stroke, e.g., in the stroke-denervated cervical spinal cord, where it was previously implicated as a potential growth-inducer. The signaling pathway includes the canonical receptor components ALK5 and SMAD3 and a downstream modulation of the cytoskeleton via protein kinase A (PKA)/SMURF1 induced downregulation of Ras homolog A (RhoA) and upregulation of PAR6. Defining the downstream signaling pathway through which TGFβ1 can induce neurite outgrowth may provide new clinical targets for future therapeutic interventions to increase compensatory sprouting, thus contributing to functional recovery.
outgrowth on the level of the cytoskeleton. This proposed mechanism of TGFβ1 signaling could underlie the observed increase in structural plasticity after stroke in vivo as suggested by the temporal and spatial expression of TGFβ1. In accordance with previous publications, this study corroborates the potential of TGFβ1 and associated signaling cascades as a target for future therapeutic interventions to enhance structural plasticity and functional recovery for stroke patients.

**Key words**: downstream signaling; neurite outgrowth; plasticity; stroke; tgfβ1

**Introduction**

Interruption of the brain’s blood supply, e.g., in case of ischemic stroke, can result in life-long disability because of the loss of neurons (Murphy and Corbett, 2009; Cramer, 2018). Most available therapeutics for stroke patients target the acute phase in the hope to protect neurons from the ischemic damage, while rehabilitation to date remains the only treatment option for chronic stroke patients (Krakauer et al., 2012; Zeiler and Krakauer, 2013; Wahl and Schwab, 2014). Both in human stroke patients as well as animal models of stroke, the spontaneous recovery observed in the weeks after the incident has been attributed in parts to structural plasticity of healthy cortical neurons and connections, e.g., horizontal neurons in the peri-infarct region or the contralateral corticospinal neurons (CSNs) in case of large strokes (Carmichael et al., 2017). In this regard, a better understanding of the molecular cues inducing, guiding and maintaining this compensatory sprouting and rewiring response is pivotal to develop novel therapeutic approaches to enhance recovery after stroke. Inflammation-derived cytokines and locally released growth factors may have a beneficial effect on the repair mechanisms by directly promoting axonal regeneration (Benowitz and Popovich, 2011; Vidal et al., 2013). In the cortex surrounding a focal stroke lesion the upregulated cytokine growth differentiation factor 10 (GDF10) was reported to enhance structural plasticity and motor recovery (Li et al., 2015). GDF10 is a member of the highly evolutionarily conserved transforming growth factor (TGF) superfamily, which also includes the TGFβ and bone morphogenetic protein (BMP) cytokine families (Weiss and Attisano, 2013; Zhang et al., 2017). A recent study highlighted the involvement of TGFβ1 signaling in neurogenesis and axonal regeneration in the peri-infarct cortex by viral knockdown of the co-receptor ALK5, leading to decreased levels of the growth associated protein GAP43 within the first two weeks after stroke, concomitant with a decrease in functional recovery (Zhang et al., 2019). A recently published transcriptomic screen showed an up-regulation of TGFβ1 in the stroke-denervated cervical hemispheric of adult mice at 28 d after a large cortical stroke. This finding suggests that TGFβ1 could be a mediator of the observed compensatory sprouting of the intact-side corticospinal tract (CST) and axon elongation/arborization in the spinal cord (Kaiser et al., 2019).

However, the mechanisms through which TGFβ1 may increase neurite outgrowth are still unclear. TGFβ1 binding to its receptor TGFβR2 can result in the formation of heteromeric complexes with two different type 1 receptors, ALK1 or ALK5, activating distinct downstream pathways often with opposing functions (Goumans et al., 2003; König et al., 2005; Stegmüller et al., 2008; Zou et al., 2009; Parikh et al., 2011; Finelli et al., 2013; Saijilafu et al., 2013; Hannila et al., 2013; Curado et al., 2014; Li et al., 2015). TGFβ1 promotes axonal outgrowth in vitro in primary neurons, including cortical neurons (Abe et al., 1996; Knöferle et al., 2010; Li et al., 2015) and blockage of the receptor TGFβR2 leads to shorter axons (Yi et al., 2010). Intriguingly, direct activation of SMAD2, the canonical downstream target of TGFβ1 signaling, reduced axonal outgrowth in some instances (Stegmüller et al., 2008; Knöferle et al., 2010). This discrepancy of axonal growth induction versus inhibition may stem from canonical versus non-canonical signaling of TGFβ1. A better understanding of the downstream signaling pathway through which TGFβ1 can induce axonal outgrowth is, therefore, urgently needed; it may also provide interesting new clinical targets for future therapeutic interventions.

In the present study, we show that TGFβ1 is transcriptionally upregulated in the region of the axotomized cervical CST and in the premotor layers of the CST-deprived spinal gray matter as early as 4 d postinjury (dpi). Using a simple but highly reproducible in vitro assay, we demonstrate that TGFβ1 treatment rescues neurite outgrowth in the growth inhibitory environment of crude spinal cord extract (SCE) through the canonical TGFβ1/ALK5 signaling axis. Further hypothesis driven pharmacological blockade studies suggest an underlying signaling mechanism

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involving a PKA-mediated phosphorylation of the E3 ubiquitin ligase SMURF1, switching its substrate preference from PAR6 to Ras homolog A (RhoA). In conclusion, we show that besides canonical transcriptional changes associated with TGF-β1, it also enhances neurite outgrowth by downregulating RhoA, the downstream signaling mediator of many CNS-associated growth inhibitory molecules. Thereby TGF-β1 has the potential to prime the neuronal cytoskeleton into a growth permissive state despite of the inhibitory CNS environment.

Materials and Methods

Animals

All animal experiments were performed with the approval of and in strict accordance with the guidelines of the Zurich Cantonal Veterinary Office. A total of n = 21 adult C57BL/6J mice (two to three months, 20–28 g, female, Charles River Laboratories) were used in this study. Only one sex was used to minimize lesion size variability and animal numbers. Animals were housed in groups of four to five under a constant 12/12 h light/dark cycle with food and water ad libitum.

Photothrombotic stroke

For all surgeries, mice were initially anesthetized using 3–4% isoflurane, transferred to a stereotactic frame (Kopf Instruments) and kept at 1–2% isoflurane throughout the surgery. Body temperature was maintained at 37°C on a heating pad. All animals received a unilateral photothrombotic stroke to lesion the right side sensorimotor cortex as previously described (Watson et al., 1985; Kaiser et al., 2019). Briefly, the skull was exposed by a midline incision of the scalp. An opaque template with a defined opening of 0.1 ml Rose Bengal (10 mg/ml in 0.9% NaCl; Sigma-Aldrich), the skull was illuminated for 10.5 min with a cold light source (Olympus, KL1500LDC, 150 W, 3000 K) placed firmly on top of the skull. Control animals were given a sham operation where necessary for 3 d.

Behavioral testing

Behavioral tests were performed before surgery (baseline) as well as 4, 7, 14, and 28 d after photothrombotic stroke (dpi) of the right motor cortex. Poststroke impairment and recovery of forelimb function was assessed using the cylinder test. Forelimb paw touches to the cylinder wall during spontaneous rearing behavior were recorded for 20 min or 30 rears in total (n = 12). Paw dragging of the affected limb was scored as the percentage of paw drags divided by total number of paw touches (Roome and Vanderluit, 2015). The horizontal ladder walk test was used as an additional, more sensitive assessment of skilled limb placement (Metz and Whishaw, 2009). Mice were habituated to the ladder run 1 d before test recordings; no further training was conducted. Three trials on a 40-cm-long ladder with irregularly spaced rungs of 1- to 2-cm distance were recorded on each testing day (n = 12). The number of foot errors was measured as the number of total misplacements (slips) divided by the total number of steps taken. All recorded steps were analyzed and no videos were excluded for the analyses to avoid bias toward stroked groups (n = 3 videos per animal per test day).

Perfusion fixation and tissue processing

All mice were terminally anesthetized with 3–5% isoflurane followed by injection of pentobarbital (300 mg/kg body weight, i.p.; Streuli Pharma AG). Animals were transcardially perfused with ice-cold Ringer’s solution (containing 105 IU/l heparin (Roche) and 0.25% NaNO2]. Brains and spinal cords were quickly dissected and snap-frozen on dry ice to preserve RNA quality. The brains were immersed in 4% PFA overnight before being transferred to 30% sucrose in phosphate buffer (PB) for cryoprotection. Samples were blindfolded from the point of tissue harvesting.

Analysis of lesion completeness

For the accurate analysis of lesion size, brain cross-sections (40 μm) were stained on-slide with cresyl violet solution for 1 min, dehydrated in a series of increasing ethanol concentrations and washed in Xylol before covering-slipping with Eukitt (Sigma-Aldrich). Brain sections at four defined landmarks (1.98, 0.98, –0.22, and –1.34 mm, in relation to bregma) were analyzed for stroke volume and depth of the cortical lesion. Average cortical stroke depth was calculated as the average of lesion depth across these four landmarks.

In situ hybridization

Sections (16 μm) of fresh frozen tissue were cut on a cryostat at –20°C and stored at –80°C. In situ gene expression was assessed using the RNAscope protocol (Advanced Cell Diagnostics, RNAscope Fluorescent Multiplex Assay) according to the manufacturer’s protocol. Briefly, slices were fixed in 4% PFA for 20 min before being hybridized to the probe (RNAscope: TGF-β1, catalog #407751), which was further amplified using the branched DNA amplification methods. Sections were counterstained with DAPI and digitalized (Zeiss, AxioScan.Z1, 200×). For analysis using ImageJ/FIJI, only slices with tears or folds were excluded; n = 3 sections per animal per group and spinal levels C5 and C6 were randomly selected. Quantification of mRNA expression was expressed as the percentage of white signal over black background in defined regions (CST in the dorsal funiculus and intermediate gray matter (iGM) of the denervated hemicord) after applying a threshold that was manually defined for five randomly selected images of the dataset and averaged.

Neurite outgrowth assay

Candidate factors were tested using a previously described neurite outgrowth assay (Maibach et al., 2020). Briefly, N1E-115 mouse neuroblastoma cells were plated
at a density of 10,000 cells/cm² in Neurobasal medium supplemented with 2% L-glutamine and 1% PenStrep to induce neuron-like differentiation. After 24 h of differentiation the cells were supplemented with crude adult rat spinal cord CHAPS extract, and candidate factors or pharmacological agents were added (Maibach et al., 2020). The following proteins and molecules were used at the indicated concentrations: 1 ng/ml TGFβ1 (R&D Systems), 1 nm TWE7197 (Selleckchem, S7530), 1 nm ML347 (Selleckchem, S7148), 10 nm SIS3 (Selleckchem, S7959), 10 nm A01 (Merck, SML1404), 10 nm Ht31 (Tocris Biotechne, 6286), and 10 nm ATM (Merck, 157201). After an additional 24-h outgrowth phase, the cells were fixed and counterstained with Coomassie solution (0.25% Coomassie Brilliant Blue R250, 50% MeOH, 10% HoAC). Images were acquired randomly.

**Immunoblotting**

Cells were washed twice in PBS on ice and lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and 50 mM Tris; pH 8) containing 2× HALT phosphatase inhibitor cocktail and 5 mM EDTA. The lysates were incubated on ice for 30 min and centrifuged at 13,000 g for 15 min at 4°C. The supernatants were collected and stored at –80°C.

The samples were prepared in Laemmli buffer (Bio-Rad) supplemented with 10% β-mercaptoethanol and denatured at 90°C for 3 min. The samples were separated on pre-cast 4–15% Mini PROTEAN® TXY™ gels (Bio-Rad) at 250 V in Tris-glycine running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS; pH 8.3). Proteins were transferred onto a 0.45μm PVDF membrane in Tris-glycine transfer buffer (25 mM Tris, 19.2 mM glycine, and 20% MeOH) for 90 min with a constant current of 300 mA. Subsequently, membranes were blocked for 1 h with 5% BSA (Sigma) in TBS-T (10 mM Tris, 150 mM NaCl, and 0.01% Tween 20; pH 7.5) and probed with primary antibodies overnight at 4°C. The membranes were washed three times in TBS-T, probed with secondary HRP-coupled antibodies (Thermo Fisher Scientific) at a concentration of 0.05–0.1 μg/ml for 1 h at room temperature (RT) and washed again three times in TBS-T. Detection was performed using SuperSignal West PICO (Thermo Scientific) or WesternBright Sirius (Advansta) chemiluminescent substrates, and images were acquired on the Gel Doc imager (Bio-Rad). Densitometry analysis was performed with ImageJ/FIJI software, and values were normalized to the housekeeping gene GAPDH or total protein and the mean value of the corresponding control group. For Western blotting, the primary antibodies are summarized in Table 1.

**Table 1: Antibodies used for Western blotting**

| Target         | Host species | Dilution | Clone          | Company                              | Catalogue number |
|----------------|--------------|----------|----------------|--------------------------------------|------------------|
| p-SMAD3 S423/425 | Rabbit       | 1:1000   | C25A9          | Cell Signaling Technology            | 9520             |
| SMAD2/3        | Rabbit       | 1:1000   | D7G7           | Cell Signaling Technology            | 8685             |
| RhoA           | Rabbit       | 1:500    | 67B9           | Cell Signaling Technology            | 2117             |
| PAR6           | Rabbit       | 1:2000   |               |                                      |                  |
| p-ERK1/2 T202/Y204 | Rabbit     | 1:1000   | 137F5          | Cell Signaling Technology            | 4695             |
| ERK1/2         | Rabbit       | 1:1000   |               |                                      |                  |
| p-S6P S235/236 | Rabbit       | 1:1000   | D57.2.2E       | Cell Signaling Technology            | 4858             |
| S6P            | Mouse        | 1:1000   | 54D2           | Cell Signaling Technology            | 2317             |
| TGFβ R2        | Goat         | 1:500    | R&D Systems    | AF-241                               |                  |
| GAPDH          | Mouse        | 1:20,000 | 6C5            | Abcam                                | ab8245           |

**RhoA assessment in growth cones**

Cells were fixed for 15 min in 4% PFA and subsequently blocked and permeabilized in blocking buffer (0.1% Triton X-100 and 10% BSA in PBS) for 1 h. Primary antibody [mouse anti-RhoA (Santa Cruz, SC-418), 1:500] was applied in 1% BSA in PBS and incubated over night at 4°C. The samples were washed three times in PBS, followed by incubation with secondary antibodies (1 μg/ml anti-mouse Cy3-coupled antibodies; Invitrogen, A10521, A10520) in 1% BSA in PBS for 1 h at RT. After washing three times in PBS, cells were counterstained with 1:100 Phalloidin Alexa Fluor 488 (Invitrogen, A12379) to visualize the actin cytoskeleton and DAPI (50 μM, Invitrogen, D3571) as a nuclear counterstain. The samples were coverslipped and imaged at 200×. For image acquisition, exposure times were kept constant and below gray scale saturation. For immunofluorescence normalization in ImageJ/FIJI, the signal in the phalloidin channel was thresholded and an area mask was created around the fluorescent object. This mask was then applied onto the RhoA pictures and the total pixel intensity within the area was measured. This value was normalized to the area of the growth cone or cell body. Similarly, a nuclear counter stain was used to define the area of the nucleus.

**Statistical analysis**

Statistical analysis was performed with Prism 7.0 (GraphPad Software). To detect differences between groups and within groups over time, two-way ANOVA followed by Dunnett’s multiple-comparisons (MC) was used. Other symbols might be used to indicate two comparisons in one graph. In bar graphs, all data are plotted as mean ± standard error of the mean (±SEM), while data were normalized to solvent controls in the neurite outgrowth assays. Dots in the behavioral graphs represent individual animals. For all neurite outgrowth assays, n = 5 biological replicates with n = 3.
technical replicates per condition. 200–400 cells per technical replicate were counted. Throughout the manuscript, *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001.

Results

TGFβ1 is endogenously upregulated at early time points in the stroke denervated hemicord

In mice, a significant degree of spontaneous re-innervation of the stroke denervated spinal cord by sprouting of contralesional CSNs can be detected 28 d after large lesions to the motor cortex (Ueno et al., 2012; Bachmann et al., 2014; Kaiser et al., 2019). At this late time point after stroke, TGFβ1 expression was found to be upregulated in the cortex (Lehrmann et al., 1995; Knuckey et al., 1996; Ata et al., 1999; Zhu et al., 2001; Doyle et al., 2010) as well as in the stroke-denervated hemicord (Kaiser et al., 2019). However, sprouting of contralesional cortical axons may be initiated much earlier in the stroke-denervated hemicord. To address whether TGFβ1 could serve as a trigger and modulator of structural plasticity in the stroke-denervated hemicord, we evaluated the expression pattern of TGFβ1 within the dorsal funiculus and gray matter at selected, early time points after stroke (2, 4, 7, and 28 dpi; Fig. 1A, B). We ensured that stroke lesions were consistent in size and location at all time points. Strokes were primarily localized to the sensory-motor cortex including the premotor regions and successfully ablated all cortical layers with little to no impact on the corpus callosum or deeper structures (Fig. 1C). This stroke model induced a behavioral deficit in the forelimbs which recovered partially over the course of four weeks as assessed by paw dragging and foot faults in the horizontal ladder task (Fig. 1D). In situ hybridization for TGFβ1 in the stroke-denervated spinal cord showed an increase of TGFβ1 mRNA at 4 dpi in both analyzed regions, the CST domain of the dorsal funiculus and iGM as well as in the iGM (Fig. 1E,F). This increased expression is transient...
in the iGM and persists up to 28 dpi in the CST, suggesting that TGFβ1 is present at time points when growth initiation and axon elongation and arborization of contralateral CSNs and other tracts occur.

**TGFβ1 rescues neurite outgrowth in inhibitory environment via canonical ALK5/SMAD3 signaling**

We first investigated the potential of TGFβ1 to rescue neurite outgrowth in a growth inhibitory environment. To model the inhibitory in vivo CNS environment, we used our previously established neurite outgrowth assay (Maibach et al., 2020). In this assay, application of crude spinal cord CHAPS extract (SCE) to the differentiated neuron-like cell line N1E-115 over a 24-h process formation phase, inhibits outgrowth in a dose-dependent manner, without affecting cell survival. As TGFβ1 was shown to increase axon length in neurons, including primary cortical neurons (Abe et al., 1996; Knöferle et al., 2010; Li et al., 2015), we first established an effect of TGFβ1 in our neurite outgrowth model. We found that already nanomolar concentrations of TGFβ1 were able to restore neurite outgrowth of IC50 SCE-treated N1E-115 cells to 80% (Fig. 2A, B).

We investigated the specific receptor complex through which the neurite outgrowth promoting TGFβ1 signaling might be transduced (Fig. 2C). While pharmacological inhibition of ALK1 had no effect on the TGFβ1-mediated rescue of neurite outgrowth in the inhibitory SCE treatment conditions, inhibition of ALK5 prevented the TGFβ1-mediated rescue (Fig. 2D, E). This suggests that the TGFβ1 signaling is propagated via the canonical TGFβR2/ALK5 signaling receptor complex. Phospho-profiling of the ALK5 downstream effector SMAD3 revealed an increased phosphorylation/activation of SMAD3 in the TGFβ1-treated conditions (Fig. 2F, G). Accordingly, pharmacological inhibition of SMAD3 prevented the TGFβ1-mediated rescue effect (Fig. 2H). Taken together, these...
results demonstrate that TGF-β1 signals via the canonical TGF-β2/ALK5/SMAD3 axis to rescue neurite outgrowth under CNS growth inhibitory conditions.

**TGF-β1 induces SMURF1-mediated downregulation of RhoA and stabilization of PAR6**

We did not observe an increase in neurite outgrowth in the absence of SCE after 24 h of TGF-β1 treatment alone (Fig. 1B) with the extremely low TGF-β1 concentration used in this study. This lack of a general growth promoting effect suggests that TGF-β1 signaling specifically interferes with and cancels the SCE-induced growth inhibitory signaling cascades. We therefore hypothesized that TGF-β1 could negatively influence the RhoA/Rho-associ- ated, coiled-coil containing protein kinase (ROCK) pathway, a key downstream signaling effector of many growth inhibitory molecules (Thiede-Stan and Schwab, 2015). One possible mechanism for such crosstalk could be the SMURF1-mediated RhoA ubiquitination which targets RhoA for degradation and thereby enhances neurite outgrowth (Wang et al., 2003; Smith et al., 2005; Sahai et al., 2007; Vohra et al., 2007; Narimatsu et al., 2009). The substrate preference of SMURF1 is modulated by PKA-dependent phosphorylation, which increases its binding affinity for RhoA relative to PAR6 (Cheng et al., 2011). In parallel to enhanced RhoA degradation, the membrane associated PAR6/PKC complex is stabilized. Both PKA activation (via association with SMAD3) and induction of a PAR6/protein kinase C (PKC) complex at the membrane have been reported as downstream effectors of TGF-β1 signaling in endothelial cells (Ozdamar et al., 2005; Wang et al., 1998; Yang et al., 2013), making it a possible mechanism for the observed rescue of neurite outgrowth by TGF-β1.

To test this hypothesis, we inhibited selected key molecules of the proposed non-canonical signaling cascade (Fig. 3A). Inhibition of SMURF1 by the blocker A01 (Cao et al., 2014) prevented the TGF-β1 elicited rescue of neurite outgrowth from SCE-induced growth inhibition (Fig. 3B). Likewise, inhibition of substrate recruiting PKA scaffolding protein A kinase anchoring protein (AKAP) by Ht31 (Kennedy and Scott, 2015) as well as pharmacological inhibition of PKC and PAR6 interaction ATM (Butler et al., 2015; Erdogan et al., 2006; Stallings-Mann et al., 2006) resulted in the loss of the TGF-β1-mediated rescue effect (Fig. 3C, D). Furthermore, TGF-β1 co-treatment with SCE resulted in a significant down-regulation of RhoA levels both globally (Fig. 3E, F) and locally in growth cones (Fig. 3H, I). This reduction of RhoA was associated with increased PAR6 levels (Fig. 3E, G), supporting the hypothesis of a TGF-β1 induced switch in SMURF1 substrate preference leading to lower levels of RhoA and higher levels of PAR6 as an underlying mechanism for the observed neurite outgrowth recovery.

Figure 3. TGF-β1 induces SMURF1-mediated downregulation of RhoA. **A**, Schematic representation of the downstream signaling pathway proposed in this study whereby activated SMAD3 activates PKA which turns P-SMURF1 to RhoA for ubiquitination; in turn, the PAR6/PKC complex is stabilized. **B**, Quantification of mean outgrowth per cell of SCE-treated N1E-115 cells with either SMURF1 inhibitor (A01) or TGF-β1 or combined treatment. **C**, Quantification of mean outgrowth per cell of SCE-treated N1E-115 cells with either AKAP inhibitor (Ht31) or TGF-β1 or combined treatment. **D**, Quantification of mean outgrowth per cell of SCE-treated N1E-115 cells with either PAR6-PKC binding inhibitor (ATM) or TGF-β1 or combined treatment. **E-G**, Representative Western blottings for RhoA and PAR6 in N1E-115 cells ± SCE and/or TGF-β1 (E) and quantification thereof (F, G). **H**, Representative pictures of growth cones stained for RhoA (red) and f-actin cytoskeleton (green). Scale bar = 10 μm. **I**, Quantification of RhoA in growth cones (GC) and cell bodies (CB), respectively; *p < 0.05, **p < 0.01, ***p < 0.001.
Discussion

We observed an upregulation of TGFβ1 mRNA at 4 dpi in the stroke-affected CST and the iGM (laminae 5–7) in the cervical spinal cord after a large stroke to the motor cortex, a time point when compensatory axonal sprouting of the CST may be triggered. This increased expression persisted in the CST area in the dorsal funiculus up to 28 dpi. In vitro neurite outgrowth analysis showed that TGFβ1 can rescue neurite outgrowth through the canonical AKT/SMAD3 signaling axis in a growth inhibitory environment. Downstream of the TGFβR2/ALK5 receptor, TGFβ1 led to the accumulation of PAR6, and negatively impacted on the RhoA/ROCK pathway by reducing the SCE-induced accumulation of RhoA. We propose an underlying signaling mechanism involving PKA-dependent SMURF1 regulation, leading to a substrate preference and an inhibitory or no effect at all (Ho et al., 2000; Do et al., 2013; Tripathi et al., 2017). Recently, TGFβ1 may also serve as a trophic factor as TGFβ1 can be secreted by astrocytes and by immune cells, thereby often functioning as an immunosuppressant (Vidal et al., 2013; Liddelow et al., 2017; Tripathi et al., 2017). TGFβ1/ALK5 signaling was associated with increased dendritic plasticity of cortical neurons, axonal sprouting of corticobulbar projections and increased motor recovery after ischemic reperfusion injury (Zhang et al., 2019).

A previous study reported an upregulation of TGFβ1 mRNA in the stroke-denervated spinal cord at 28 dpi (Kaiser et al., 2019), suggesting that TGFβ1 might serve as a local tissue-derived pro-regenerative cue for re-innervating axons after cortical stroke. Here, we observed an increase in TGFβ1 mRNA as early as 4 dpi. While we found a transient increased expression of TGFβ1 in the iGM, this increase persisted in the dorsal funiculus in the CST area. Interestingly, this temporal and spatial expression profile matches the previously described activation of microglia and infiltration of macrophages (Kaiser et al., 2019), suggesting that these cells may be the predominant source of TGFβ1 in the stroke-denervated spinal cord. This interpretation is in line with a previous finding of increased TGFβ1 expression in microglia/macrophages in the stroke penumbra early after middle cerebral artery occlusion (Doyle et al., 2010).

We addressed the potential downstream pathways of TGFβ1 leading to increased neurite outgrowth under growth inhibitory conditions. We conducted our studies using an in vitro model the neuroblastoma-derived cell line N1E-115 treated with crude SCE (Maibach et al., 2020). While this model lacks the direct translational impact of primary cell cultures, it ensures a high reproducibility between studies that primary cultures fail to provide owing to an often large batch-to-batch variability and contamination with non-neuronal cells. High sensitivity and reproducibility are a crucial prerequisite for a routine assay that is used for therapeutic compound screening. By using the well characterized neuron-like N1E cells, we were able to avoid non-neuronal cell contamination by, e.g., astrocytes, which might serve as a primary source for TGFβ1 and thus interfere with our results in this study. An additional advantage of this assay is the ability to distinguish effects of the treatment on neurite outgrowth directly versus neuronal cell death as counted by number of cells. Treatment of N1E cells with SCE did not lead to an increase in cell death, but to a stunted neurite outgrowth with cells portraying a “panning” like phenotype. Co-treatment with TGFβ1 led to a robust increase in neurite outgrowth under these inhibitory conditions. Moreover, the neurite growth inhibitory effects of SCE in the N1E cultures were highly comparable to those previously published in primary cortical or hippocampal neurons, and the TGFβ1-mediated rescue of neurite outgrowth from SCE-induced growth inhibition in our in vitro model of N1E-115 cells was dependent on ALK5 and SMAD3, as was shown to be the case for cortical neurons in vivo (Zhang et al., 2019).

We then went on to pharmacologically block selected downstream targets of TGFβ1/ALK5 signaling to identify potential effects on neurite outgrowth in this model. By applying the candidate factors in a well-defined time frame after initial neurite outgrowth and in combination with inhibitory SCE, we were able to address post-translational signaling mechanisms, including phosphorylation and ubiquitination. While these studies allow us to address the direct effects of pharmacological interventions, we did not address effects on a transcriptional level in this study.
Application of SCE was shown to increase RhoA levels both in the cell body as well as in the growth cone. RhoA activity is associated with neurite retraction in response to growth cone collapsing agents (Feltrin and Pertz, 2012; Jeon et al., 2012). Pharmacological blockade of RhoA or the downstream effector Rho-kinase (ROCK) abolished the inhibitory effects of SCE, specifically also of its active ingredients Nogo-A and MAG (Niederöst et al., 2002). TGFβ1 treatment was associated with a downregulation of RhoA back to baseline levels (control group without SCE treatment), indicating that TGFβ1 signaling might have led to a decreased response to the inhibitory molecules of SCE such as Nogo-A or MAG.

We further saw an accumulation of PAR6 protein in the TGFβ1-treated N1E-115 cells, suggesting increased protein stability or synthesis resulting from the TGFβ1 treatment. Previous studies have described TGFβ1-mediated regulation of proteasomal processes in neuronal cells (Tadlock et al., 2003; Knöferle et al., 2010). Along these lines, we found that the TGFβ1-mediated rescue of neurite outgrowth depended on the E3 ubiquitin ligase SMURF1 as well as PKC/PAR6 interaction. In various cell types, TGFβ1 signaling induces cellular polarity by recruitment and activation of the PAR6/PKC complex to TGFβ2 (Ozdamar et al., 2005). Other studies suggested that this complex relocates SMURF1 to the membrane, where it induces RhoA degradation (Wang et al., 2003; Sahai et al., 2007; Narimatsu et al., 2009) and neurite outgrowth (Bryan et al., 2005; Vohra et al., 2007).

Notably, in our study, the TGFβ1 induced neurite outgrowth was also dependent on PKA activation. Previous studies have shown that PKA is a downstream target of TGFβ1 and is associated with SMAD proteins (Wang et al., 1998; Yang et al., 2013). PKA-mediated phosphorylation of the ubiquitin ligase SMURF1 in the RhoA interaction domain was shown to increase SMURF1 affinity for RhoA relative to PAR6, thereby contributing to axon formation in vitro and neuronal polarization in vivo (Cheng et al., 2011). It is possible that TGFβ1 signaling may also influence additional Rho-like GTPases such as Rac and Cdc42 and effectively regulate neurite outgrowth through additional signaling pathways (Zhang, 2009). Based on the present data, we propose that TGFβ1 enhances axonal outgrowth via stabilization of PAR6 and degradation of RhoA via a PKA/NF-kB-dependent mechanism. These results can guide future experiments to analyze these mechanisms in vivo and in particular in CSNs after a cortical stroke.

Endogenous TGFβ1 levels were shown to be increased in early phases after stroke, within the critical period of plasticity (Doyle et al., 2010). While the present and other studies suggest that prolonged treatment with TGFβ1 or a downstream effector of the TGFβ1/ALK5 signaling pathway may be used to enhance plasticity and extend its critical window after stroke (Li et al., 2015; Zhang et al., 2019), a systemic delivery of TGFβ1 would bring about unwanted negative side-effects owing to the plethora of functions of TGFβ1 as an inflammation related molecule (Akhurst and Hata, 2012). It is thus necessary to understand the specific signaling mechanisms by which TGFβ1 mediates structural plasticity and axonal outgrowth, both in vitro and in vivo, to develop a successful therapeutic agent.

The present study investigates a potential signaling mechanism through which TGFβ1 induces neurite outgrowth in a growth inhibitory environment in vitro and possibly compensatory axonal sprouting in vivo. This signaling pathway includes the canonical receptor components ALK5 and SMAD3 and a downstream activation of the cytoskeleton via PKA/SMURF1 induced downregulation of RhoA and upregulation of PAR6.

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