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# The Tenascin-C-Derived Peptide VSWRAPTA Promotes Neuronal Branching Via Transcellular Activation of the Focal Adhesion Kinase (FAK) and the ERK1/2 Signaling Pathway In Vitro

Marvin Jarocki[1] · Omar Sallouh[2] · Ralf Weberskirch[2] · Andreas Faissner[1]

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## Abstract

The central nervous system (CNS) of mammals has a limited regeneration capacity after traumatic events, which causes chronic functional disability. The development of biomaterials aims at providing support for the regeneration process. One strategy integrates peptides that mimic functional domains of extracellular matrix (ECM) or cell adhesion molecules with synthetic polymers designed to present growth-supporting cues to the neuronal microenvironment. Thus, small peptide sequences originating from molecules of the ECM may serve as promising bio-additives, acting as artificial matricryptins to gear cellular processes. The glycoprotein tenascin-C (Tnc) is a major constituent of the ECM of the developing brain and persists in the neurogenic regions of the adult CNS. It is a multimodular glycoprotein that comprises distinct domains with neurite growth promoting and axon growth repulsing properties. In the present study, the novel peptide motif VSWRAPTA that is encoded in the neurite growth promoting 6th fibronectin type III repeat close to the alternative splice site of Tnc was tested for its effects on neuron differentiation. When this newly synthesized biomimetic peptide was added to cultures of embryonic cortical neurons it significantly promoted the outgrowth of neurites. The neuron differentiation supporting effect was thereby associated with the trans-cellular activation of the focal adhesion kinase (FAK) and the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway. Cortical neurons supplemented with the Tnc peptide displayed a dose-dependent increase in neurite outgrowth that saturated at a peptide concentration of 50 μg/ml (56.4 mMol/l). The analysis of neuron morphology revealed that neurite branching rather than fiber length was stimulated by the Tnc peptide. Therefore, we predict that the analyzed peptide motif of the 6th constitutively expressed FNIII domain of the Tnc molecule might be a major contributor for neurite outgrowth and guiding events in the native CNS microenvironment. In conclusion, the Tnc-derived VSWRAPTA peptide may represent a promising tool to spike regeneration supportive microenvironments.

**Keywords** Axon growth and guidance · Axon regeneration · Extracellular matrix · Cortical neuron · Signal transduction · Neurite sprouting and branching · Tenascin-C

## Introduction

The recruitment and functional integration of neurons represents a pivotal event during neural development that is decisive for the formation of functional neural networks. The reconstruction of these networks upon trauma or disease is a prime objective of regenerative medicine. Despite considerable progress in recent years, the corresponding processes still remain insufficiently understood. In this context, the extracellular matrix (ECM) plays an important role by mediating cellular fate decisions, cell migration, and network formation [1–3].

Axon growth and guidance is a key process that is precisely regulated by intrinsic and extrinsic factors [4]. During embryonic development, the glycoprotein of the ECM tenascin-C (Tnc) is a major component that is known to affect neurite outgrowth and guidance by specific fibronectin type III (FNIII) domains [5–8]. Up to 27 different splice variants of *Tnc* have been detected during murine cerebral development, leading to the hypothesis that Tnc isoforms might contribute to the specification of neuronal microenvironments [2, 9, 10].

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✉ Andreas Faissner
andreas.faissner@ruhr-uni-bochum.de

[1] Department of Cell Morphology and Molecular Neurobiology, Ruhr-University Bochum, Universitaetsstr. 150, 44801 Bochum, Germany

[2] Faculty of Chemistry and Chemical Biology, TU Dortmund, Otto-Hahn Str. 6, 44227 Dortmund, Germany

In the last decades, studies with reference to the different splice variants of Tnc revealed multiple functions of Tnc that result from variable combination and integration of different alternatively spliced FNIII domains into the Tnc molecule (for a compact overview, see [11]). During development of the murine neocortex, Tnc is predominantly expressed within the subventricular zone, the major germinal area in which neuronal progenitors are generated. These progenitors subsequently migrate to terminal positions in deeper cortical layers to form complex networks for proper signal transduction [12–14]. For example, Tnc contributes significantly to the correct formation and integration of thalamic and cortical axons within the developing cerebrum by distinct Tnc FNIII repeats [15]. Further, perturbation experiments led to the conclusion that separate FNIII repeats can exert cooperative effects, suggesting that cells of the ventricular zone do regulate axonal projections through specific locally restricted deposition of the Tnc molecules [15].

Several reports indicated that stimulation of neurite outgrowth by Tnc is dependent on the distinct alternatively spliced FNIII domain D of the Tnc molecule (TNfnD) [6–8]. Within the FNIII domain D, the conserved sequence of eight consecutive amino acids VFDNFVLK, turned out to be essential for facilitating the process of neurite elongation via interaction with the α7β1 integrin [16, 17]. When integrated into amphiphilic nanofiber gel substrates, this peptide sequence significantly promotes neurite outgrowth and migration of cells derived from neurosphere cultures [18]. Recently, we have described the novel peptide sequence VSWRAPTA derived from the 6th FNIII domain close to the neurite outgrowth-promoting region that promotes motility of tumor cells [19]. In the present study, we highlight the neurite growth-promoting property of this peptide. We provide evidence that the synthetic octapeptide increases neurite formation and branching of embryonic cortical neurons by transcellular activation of the focal adhesion kinase (FAK) and the downstream extracellular signal-regulated kinase 1/2 (ERK1/2) pathway. Thereby, it may provide a novel tool to spike regeneration supporting polymers.

## Material/Methods

### Immunological Reagents

For specific immunocytochemical detection following primary immunological reagents with respective dilutions were used. Monoclonal antibodies against the class III β-tubulin epitope (1:300; mouse IgG2b; clone SDL3D10; Sigma-Aldrich, St. Louis, USA) for specific identification of neurons, O4 (1:30; mouse IgM; clone 81 [20] to mark oligodendrocytes, nestin (1:500; mouse IgG1; Merck Millipore, Billerica; USA) for detection of neural precursor cells and a polyclonal antibody against the glial fibrillary acidic protein epitope (GFAP, 1:300; rabbit IgG; Dako GmbH, Hamburg, Germany) to mark astrocytes, were used. The nuclei were stained, using the DNA intercalating bisbenzimide dye Hoechst 33258 (Sigma-Aldrich, St. Louis, USA) in a concentration of 1 μg/ml. Species-specific secondary antibody solutions, either coupled with Cy3 (1:500; goat anti-mouse IgG; Dianova GmbH, Hamburg, Germany), Alexa Fluor 488 (1:250; goat anti-mouse IgG + IgM; Dianova GmbH, Hamburg, Germany), or Cy2 (1:250; goat anti-mouse IgM; Dianova GmbH, Hamburg, Germany), were used to detect primary antibody marked epitopes. Western blot analysis was carried out using following primary immunological reagents, with respective dilutions. Antibodies against α-tubulin (1:5000, Sigma-Aldrich Corp., St. Louis, USA), p42/44 MAPK (1:1000, Cell Signaling Technology Corp., Cambridge, UK), total ERK1/2 (1:1000, Santa Cruz Biotechnology Inc., Dallas, USA), as well as focal adhesion kinase (FAK) (1:1000, Cell Signaling Technology Corp., Cambridge, UK), and pFAK (1:1000, Cell Signaling Technology Corp., Cambridge, UK) were used. Species-specific horseradish peroxidase (HRP)-coupled secondary antibodies (1:5000, all Dianova GmbH, Hamburg, Germany) were used for detection of primary antibody-coupled protein epitopes.

### Dissection and Cultivation of Acutely Dissociated Neurons

All cell culture experiments presented in this work are based on E12.5 embryonic neural tissue isolated from time-mated NMRI wild-type albino mice, with stage determination following the Theiler criteria [21]. Mice had free access to water and food ad libitum on a standardized 12 h circadian rhythm. The present study was carried out in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EU) for care of laboratory animals. All efforts were made to reduce the number of animals used. The animals were killed by cervical dislocation to avoid extensive stress. After killing the timed pregnant mouse, the abdomen was sterilized with 70% plain ethanol (EtOH) prior to the dissection of the uteri. Afterwards, the uteri were isolated and subsequently transferred into a culture dish (Thermo fisher scientific, Waltham, USA) containing sterile Minimum Essential Media Eagle (MEM) solution (Sigma-Aldrich, St. Louis, USA). The embryos were freed from the uteri and subsequently decapitated. Thereafter, brains were isolated and neocortices were dissected. After carefully removing circumjacent meninges, the neocortical tissue was separated from ganglionic eminences and hippocampal anlagen. The tissue was collected in a precooled 2 ml reaction tube containing 1 ml sterile MEM solution (Sigma-Aldrich, St. Louis, USA). Enzymatic digestion was then initiated, by addition of 1 ml

sterile dissociation solution containing MEM (Sigma-Aldrich, St. Louis, USA) supplemented with 30 U/ml papain (Worthington, Lakewood, USA), 40 μg/ml DNase I (Worthington, Lakewood, USA), and 240 μg/ml L-cystein (Sigma-Aldrich, St. Louis, USA). Digestion was carried out for 30 min at 37 °C. To stop the process of digestion, 1 ml of digestion solution was carefully aspirated and replaced by an equivalent volume of ovomucoid (L15 medium (Sigma-Aldrich, St. Louis, USA) supplemented with 0.1% (*w*/*v*) soybean trypsin inhibitor, 0.005% (*w*/*v*) bovine serum albumin (BSA) V (Sigma-Aldrich, St. Louis, USA), and 0.1% DNase I (Worthington, Lakewood, USA)). The predigested tissue was gently triturated, and the resulting single cell suspension was centrifuged for 5 min at 200×*g*. After aspiration of the supernatant, the cells were resuspended in neuron medium (MEM (Sigma-Aldrich, St. Louis, USA) supplemented with 2% (*v*/*v*) B27 (Life technologies, Carlsbad, USA), 0.01% (*w*/*v*) ovalbumin (Sigma-Aldrich, St. Louis, USA)), 100 U/ml penicillin/100 μg/ml streptomycin (Life Technologies, Carlsbad, USA), 10 mM sodium pyruvate (Sigma-Aldrich, St. Louis, USA), and subsequently plated on poly-L-ornithine (10 μg/ml, Sigma-Aldrich, St. Louis, USA) precoated culture dishes (25,000 cells per 78.54 $mm^2$ (∅ 10 mm) or 500,000 cells/2827.43 $mm^2$ (∅ 60 mm), Thermo Fisher Scientific, Waltham, USA). Neurons were cultivated for 3 days in vitro at 37 °C and 6% $CO_2$, either in presence of peptide (at concentrations of 10, 50, or 100 μg/ml), or without additional peptide supplementation. We made a strong effort to establish a serum-free culture system of embryonic cerebral cortex-derived neurons because either the presence of glial cells or the addition of serum to the culture medium impact on the differentiation of neurons in various ways. By omitting these components, we focused as stringently as possible on the intrinsic properties of the VSWRAPTA peptide with regard to neuronal differentiation.

## Generation of Embryonic Neural Stem Cells Using the Neurosphere Culture System

The neurosphere culture system represents a convenient way to generate larger quantities of neural stem cells for further cell culture experiments. Here, sufficient amounts of neural stem cells were obtained by dissection of neocortical tissue and subsequent cultivation as free-floating neurospheres [22, 23]. Embryonic neocortex was prepared and dissociated as described in the previous section (see above). After digestion, manual trituration, and centrifugation, the resulting cell pellet was resuspended in 1 ml neurosphere medium, containing Dulbecco's modified Eagle's medium (DMEM) and nutrient mixture F12 in a ratio of 1:1 (both Sigma-Aldrich, St. Louis, USA), 2% (*v*/*v*) B27 (Life Technologies, Carlsbad, USA), 1% (*v*/*v*) L-glutamine (Life Technologies, Carlsbad, USA), and 1% (*v*/*v*) penicillin/streptomycin (Life Technologies, Carlsbad, USA). Next, the cell number was determined manually via analysis of 10 μl cell suspension in a Neubauer counting chamber. To ensure optimal culture conditions, cells were plated at a density of 100,000 cells/ml in appropriate cell culture flasks (T-25 flask, 25 $cm^2$, Sarstedt, Nümbrecht, Germany) for 6 days at 37 °C and 6% $CO_2$, in presence of 20 ng/ml epidermal growth factor (EGF) (Preprotech GmbH, Hamburg, Germany), 20 ng/ml fibroblast growth factor (FGF) (Peprotech GmbH, Hamburg, Germany), and 0.5 U/ml heparin (Sigma-Aldrich, St. Louis, USA). During cultivation, agitation was strictly avoided to prevent nonclonal neurosphere formation. After 6 days of cultivation, neurospheres of about 200 μm in diameter had formed.

## In Vitro Cultivation of Glia Cell Containing Cultures from Embryonic Neural Stem Cells

For the analysis of neurons under influence of the Tnc peptide in presence of glia cells, neural stem cell cultures under differentiating conditions were used. To this end, neurospheres were centrifuged for 2 min at 200×*g*. After discarding the supernatant, the cell clusters were carefully resuspended in 500 μl 0.05% (*w*/*v*) trypsin/ethylenediaminetetraacetic acid (EDTA) (Life Technologies, Carlsbad, USA) and subsequently incubated in a prewarmed water bath at 37 °C for 5 min. To end the digestion process, an additional volume of 500 μl ovomucoid (L15 medium (Sigma-Aldrich, St. Louis, USA) supplemented with 0.1% (*w*/*v*) soybean trypsin inhibitor, 0.005% BSA (*w*/*v*, Sigma-Aldrich, St. Louis, USA), and 0.004% (*w*/*v*) DNase I (Worthington, Lakewood, USA)) was added to the cell suspension, followed by gentle manual trituration. The resulting single cell suspension was centrifuged again for 5 min at 200×*g* and overlaying digestion solution aspirated. Finally, the cells were transferred into differentiation media, containing DMEM and nutrient mixture F12 in a ratio of 1:1 (both Sigma-Aldrich, St. Louis, USA) with additional 2% (*v*/*v*) B27 (Life Technologies, Carlsbad, USA), 1% L-glutamine (Life Technologies, Carlsbad, USA), 1% (*v*/*v*) penicillin/streptomycin (Life Technologies, Carlsbad, USA), and 1% (*v*/*v*) fetal calf serum (Sigma-Aldrich, St. Louis, USA). Afterwards, the cells were applied to poly-L-ornithine (10 μg/ml, Sigma-Aldrich, St. Louis, USA) precoated culture dishes (25,000 cells per 78.54 $mm^2$ (∅ 10 mm) Thermo Fisher Scientific, Waltham, USA), and incubation with or without additional Tnc peptide (50 μg/ml) was performed for 3 div at 37 °C and 6% $CO_2$.

## Cultivation of Neurons Under Nonstimulating Conditions for Specific FAK and ERK1/2 Assessment

In order to analyze specific intracellular signal transduction molecule activation upon treatment with the biomimetic Tnc peptide, neurons were cultivated as described

previously for 3 days in vitro, in the absence of the peptide. After initial cultivation, cultures were subjected to nonstimulating conditions 6 h prior to peptide administration. According to Xiao [24], cultivation of cells in MEM in the absence of additional serum or additives like B27 lowers the basal metabolism and thereby basal FAK and ERK1/2 signaling. Therefore, neuronal cultivation medium was carefully aspirated, and cultures were washed once with prewarmed MEM (Sigma-Aldrich, St. Louis, USA) solution. Finally, MEM (Sigma-Aldrich, St. Louis, USA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (both Life Technologies, Carlsbad, USA), as well as 10 mM sodium pyruvate (Sigma-Aldrich, St. Louis, USA) was applied to the cultures. After 6 h of incubation at 37 °C and 6% $CO_2$, biomimetic peptide was administered at concentrations of 50 μg/ml to the cultures. At specific time points, total protein was isolated from neuronal cultures to monitor changes in the phosphorylation level of FAK and ERK1/2. To this end, cultures were washed carefully once with phosphate-buffered saline solution (PBS), prior to protein isolation via addition of 150 μl (500,000 cells/dish (∅ 60 mm)) precooled protein lysis buffer (60 mM *N*-octyl-β-D-glucopyranoside, 50 mM sodium acetate, and 50 mM Tris (pH 8.5)).

## Immunocytochemical Detection of Neural Cell-Specific Epitopes

Detection of neural cell-specific epitopes was carried out by use of immunocytochemical detection procedures as described previously [22]. To this end, media of neuronal cultures was aspirated at first, before cells were washed and blocked twice in prewarmed KRH/A solution (Krebs-Ringer HEPES solution (KRH), supplemented with 0.1% (*w*/*v*) BSA (Sigma-Aldrich, St. Louis, USA)). The identification of the membrane-associated epitope O4 was carried out before fixation, diluting the antibody in KRH/A and incubation for 1 h at room temperature (RT). After aspiration of the immunological reagent and washing three times with KRH/A, the cultures were fixed in PBS containing 4% (*w*/*v*) paraformaldehyde (PFA; Carl Roth GmbH, Karlsruhe, Germany) for 10 min at RT. Subsequently, the cells were washed again three times with PBT-1 solution (PBS, supplemented with 1% (*w*/*v*) BSA and 0.1% (*v*/*v*) Triton X-100 (both, Sigma-Aldrich, St. Louis, USA)) for 5 min, to eliminate residual PFA and to permeabilize cellular membranes. Simultaneously, primary antibody solutions were diluted in PBT-1 solution and applied to the cells for 1 h at RT. After incubation, the wells were freed from primary antibody solution and again washed three times with PBS/A (PBS supplemented with additional 0.1% (*w*/*v*) BSA (Sigma-Aldrich, St. Louis, USA)). Appropriate fluorophore-coupled secondary antibodies, in combination with nuclei staining solution (Hoechst 33258, Sigma-Aldrich, St. Louis, USA) were diluted in PBS/A and applied to the cells for 1 h at RT. Thereafter, the solution was aspirated, cells were washed three times with PBS, and finally covered with PBS/glycerol (1:1) and a glass coverslip. Photomicrographs of immunocytochemically labeled cells were taken at a fluorescence microscope (Zeiss Axioplan Imaging 2) and appropriate software (AxioVision, ver. 4.8.2 SP3). Micrographs with images of the cultures were handed to a third person, pooled, randomized, and given to the investigator for further analysis. Quantitative assessment was performed under blinded conditions.

## Assessment of Neurite Outgrowth

For further neurite outgrowth and branching analysis, ImageJ software (Release 1.51d, Wayne Rasband, National Institutes of Health, Bethesda, Maryland) with plugin extensions Neurite Tracer [25] (Montreal Neurological Institute, Montreal, Canada, alyson.fournier@mcgill.ca) and NeuronJ (release 1.4.3 [26], Eric Meijering, Erasmus University Medical Center, Rotterdam, Netherlands, meijering@imagescience.org) were used according to authors' instructions. Thereby, Neurite Tracer plugin was used for semi-automated analysis of neurite and neuronal network formation, while NeuronJ represents a tool for manual assessment of individual neurites.

## Real-Time Quantitative Polymerase Chain Reaction

Gene expression analysis was performed using RT-qPCR analysis of cDNA, reversely transcribed from whole RNA samples as described previously [27]. To this end, total RNA was isolated from neuronal cultures (GenElute Mamalian Total RNA Miniprep Kit, Sigma-Aldrich Corp., St. Louis, USA) and transcribed into cDNA (First Strand cDNA Synthesis Kit, Thermo Fisher Scientific Inc., Waltham, USA) according to the manufacturer's instructions. RT-qPCR analysis was performed using the Fast Essential DNA Green Master Kit (Hoffmann-La Roche AG, Basel, Switzerland) and the LightCycler® 96 system with appropriate software (Light Cycler® 96 Software 1.1.0.1320, Hoffmann-La Roche AG, Basel; Switzerland) for specific Cq value determination of different target and housekeeping genes (Table 1). Further, relative expression of target genes and statistical evaluation was carried out averaging the target gene Cq values with reference to three housekeeping genes (according to MIQE guidelines [28]) 18s, β-actin, and cyclophilin, using REST 2009 software [29]. Following primers were used for specific target gene analysis and are based on suggestions of the Roche Universal ProbeLibrary Assay Design Center for optimal real-time PCR assays (Table 2).

**Table 1** RT-qPCR protocol for analyses of specific neurite outgrowth associated genes

| RT-qPCR protocol for LightCycler® 96 device (dye: SYBR Green I) | | | |
|---|---|---|---|
| Temp. (°C) | Ramp (°C/s) | Duration (s) | Acquisition mode |
| Preincubation | | | |
| 95 | 4.4 | 600 | None |
| 3-Step amplification (45 cycles) | | | |
| 95 | 4.4 | 10 | None |
| 60 | 2.2 | 30 | None |
| 72 | 4.4 | 10 | Single |
| Melting | | | |
| 95 | 4.4 | 10 | None |
| 65 | 2.2 | 60 | None |
| 97 | 0.2 | 1 | 5 readings/°C |
| Cooling | | | |
| 37 | 2.2 | 30 | None |

Standard RT-qPCR protocol based on suggestion of manufacturer for SYBR Green I-mediated analysis (Fast Essential DNA Green Master Kit; Hoffmann-La Roche AG, Basel, Switzerland)

## Protein Isolation and Detection Procedure Using SDS-PAGE and Western Blot Analysis

Protein separation was performed by SDS-PAGE [30]. Protein samples were collected, and concentrations were determined as described previously [31]. To this end, media were aspirated, and cells were washed twice with 1× PBS. Following to that, 150 μl of ice-cold lysis buffer replenished with 1 mM PMSF and 1 μg/ml aprotinin (both Sigma-Aldrich Corp., St. Louis, USA) was applied to the cultures, cells were harvested by scraping with appropriate rubber policemen and collected in a chilled 1.5 ml reaction tube. Cell debris containing protein suspension was centrifuged at 16,100×*g* and 4 °C for 10 min. The protein containing supernatant was collected and protein concentrations were determined with the BCA Protein Assay Reagent kit (Thermo Fisher Scientific Inc., Waltham, USA) according to manufacturer's instructions. After adjusting protein concentrations, probes were supplemented with 4× sample buffer (containing 250 mM Tris/HCl (pH 6.8), 0.25% (*w*/*v*) bromophenol blue, 40% (*v*/*v*) glycerol, 20% (*v*/*v*) 2-mercaptoethanol, 9.2% (*v*/*v*) sodium dodecyl sulfate (SDS)) and denatured for 5 min at 95 °C. Subsequently to thermal denaturation, probes were chilled and applied to the stacking slots of casted 12% (*v*/*v*) SDS containing polyacrylamide gels. Thereafter, electrophoretically separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Carl Roth GmbH, Karlsruhe, Germany). To this end, the protein containing gels were placed within a semi-dry blot chamber (Hoefer Inc., Holliston, USA), on top of three transfer buffer wetted Whatman filter papers (Bio-Rad Inc., Hercules, USA) and a methanol-activated PVDF membrane, topped by another three transfer buffer-wetted Whatman papers. By applying a specific current of 1.5 mA/cm$^2$ for 1.5 h, proteins were transferred to the PVDF membranes. Following to the blot process, the membranes were blocked for 1 h at RT in 5% (*w*/*v*) semi-skimmed milk powder (Heirler Cenovis GmbH, Radolfzell am Bodensee, Germany) containing tris-buffered saline (TBS) with 0.05% (*v*/*v*) Tween-20 solution (TBST; Carl Roth GmbH, Karlsruhe, Germany), before epitope detection with appropriate primary antibodies was applied. Primary antibody solutions were diluted in aforementioned blocking solution and incubated on top of the membranes over night at 4 °C under constant agitation. After incubation, the primary antibody solution was aspirated and the membranes were

**Table 2** List of primers used for RT-qPCR

| Gene | Forward primer | Reverse primer | Annealing temp. (°C) | Amplicon size (nt) | Reference (Roche UPLADC) |
|---|---|---|---|---|---|
| Cntn3 | (1409) tctggaagaag ggagacatga (1429) | (1460) tgattttgagtc ctccatcattta (1483) | 60 | 75 | NM_008779.2 |
| Ctgf | (1013) tgacctggagga aaacattaaga (1035) | (1103) agccctgtatg tcttcacactg (1124) | 60 | 112 | NM_010217.2 |
| Cux2 | (3635) tggagaagc ttcgggacat (3653) | (3676) catagcggc gcttcagata (3694) | 60 | 60 | NM_007804.2 |
| FoxP2 | (520) gacccctgga gaggactaaag (540) | (588) cttggagtgga gtcctaagttca (610) | 60 | 91 | NM_053242.4 |
| Itga7 | (900) aaaactttgg accctgctga (919) | (966) ccagaatcga tggagaaacc (985) | 60 | 86 | NM_008398.2 |
| ItgaV | (1710) ggtgtggatc gagctgtclt (1729) | (1751) caaggccag catttacagtg (1770) | 60 | 61 | NM_008402.2 |
| Itgb1 | (661) ctgcttctaaaat tgagatcagga (684) | (729) tccataaggtagta gagatcaataggg (755) | 60 | 95 | NM_010578.2 |
| Itgb3 | (2185) gtgggagg gcagtcctcta (2203) | (2227) caggatatca ggacccttgg (2246) | 60 | 62 | NM_016780.2 |
| 18s RNA | (1623) gcaattattc cccatgaacg (1642) | (1671) gggacttaatc aacgcaagc (1690) | 60 | 68 | NR_003278.2 |
| Cyclophilin | (3451) aaggatggca aggattgaaa (3470) | (3535) ctttaagcaat tctgcctgga (3555) | 60 | 105 | ENSMUSTOO 000161460.1 |
| β-Actin | (414) ctaaggccaa ccgtgaaaag (433) | (498) accagaggca tacagggaca (517) | 60 | 104 | NM_007393.5 |

The primer pairs used in this study were generated and ordered according to the Roche Universal ProbeLibrary Assay Design Center for optimal real-time PCR assays

washed three times for 15 min in TBST solution. Species-specific HRP-coupled secondary antibody were diluted in blocking solution and subsequently applied to the membranes. For 1 h, membranes were incubated at RT and afterwards washed three times with TBST solution. An additional washing step with TBS was applied to eliminate residual detergents. Detection of marked epitopes was carried out using the Clarity™ Western ECL Substrate (BioRad Lab. Inc., Hercules, USA), according to the manufacturer's instruction. Detection and documentation of the resulting chemiluminescence signal was done in a microchemi detection chamber (DNR Bio-imaging Systems Ltd., Jerusalem, Israel), with subsequent densitometric analysis of the chemiluminescence signals using TotalLab Quant software (release 11.4, Newcastle upon Tyne, UK) according to given instructions for Western blot analysis. Here, relative intensity of the chemiluminescence signal of a target protein (pFAK or pERK1/2) was measured and averaged firstly in relation to the respective unphosphorylated target protein (FAK or ERK1/2) and next to the base chemiluminescence signal of the target protein before peptide addition. To further support comparability of the different probes visually (besides determination of Protein concentration), Western blot analysis with antibodies detecting α-tubulin were performed and presented.

### Peptide Monomer Synthesis

Peptide monomer (PM) synthesis was carried out using the Fmoc-stategy for automated peptide synthesis as previously described [22]. PMs were provided in a lyophilized state for better handling and stored at − 32 °C. Upon use, PMs were dissolved and aliquoted in $ddH_2O$ according to Peptide Storage and Handling Guidelines (Genescript, Piscataway, USA) and further applied to cell cultures, as previously described [19].

### Statistical Evaluation and Data Acquisition

Unless otherwise declared, all data represent at least three biological replicates ($N$), with technical replicates ($n$) as indicated in the respective figure legend. Statistical data analysis was carried out using SPSS software (release 20.0.0, IBM, Armonk, USA) for assessment of significance levels. Thereby two-paired standard Student's $t$ test was used for data of two groups values, whereas data from more than two independent groups were analyzed by analysis of variance (ANOVA) with additional post hoc testing. Information about used post hoc test is given in the figure legends. For neurite outgrowth analysis in cultures, relative neurite extension values were scored in order to correct for variations between independent experiments. Individual length values $L_i$ were divided by the average length of the control culture ($L_{ctrl}$; $R_i = L_i/L_{ctrl}$ and expressed as ratio $R_i$). Alternatively, relative increase or decrease of a given parameter $P_i$ was calculated and given as percent (%). Therefore, the average value of the parameter for control conditions ($P_{ctrl}$) was calculated and compared with experimental values ($P_{test}$) using the equation ($\% = (P_{test} - P_{ctrl}) / P_{ctrl}) \times 100$). Thereby, the relative values of independent experiments could be pooled and tested for statistical significance assessment. Similarly, for Western Blot analysis of ERK1/2 and FAK (Fig. 4), values of treated cultures were normalized to values obtained in control cultures.

## Results

### Cortical Neuronal Cultures from E12.5 Embryonic Tissue

In previous studies, we had shown that the tenascin-C (Tnc)-derived peptide VSWRAPTA promotes the motility of tumor cells [19]. When we added the peptide to cultures of neural stem cells from embryonic brain stimulated to differentiate by the addition of serum [23], we observed enhanced neurite outgrowth (supplementary Fig. 1). Differentiating neural stem cell cultures contain glial cells and serum in addition to neurons, which both are known to promote neurite outgrowth. In order to disentangle the specific contribution of the peptide per se, we have relied on the most accurately controlled conditions of defined media. Therefore, we considered a pure neuronal culture as the prerequisite for experimental approaches regarding putative effects of the Tnc peptide on neurite outgrowth. For this purpose, we established a protocol for primary cortical neuron cultures from embryonic tissue of E12.5 mice. Acutely dissociated cells were plated on poly-L-ornithine-coated dishes and cultivated in presence of neuron-promoting media at 37 °C and 6% $CO_2$, without additional feeder layer. The neurons could be cultivated up to 14 days (Fig. 1d). Due to progressive neurite elongation in vitro, estimation of neurite promotive effects were most reliable after 3 days of cultivation (Fig. 1a). Immunocytochemical stainings against epitopes of the three major lineages of the CNS, classified by class III β-tubulin-identifying neurons, GFAP marking astrocytes, O4 for immature oligodendrocytes, and additional neural stem cell marker nestin, attested the quality and large fraction of neurons within the culture. Thus, 82.51 ± 2.09% of cells present in the cultures, exhibited specific neuronal properties (by expression of the class III β-tubulin epitope), while 13.20 ± 2.09% of the plated cells remained in an undifferentiated state (visualized by nestin epitope staining). Only a minority of 0.06 ± 0.04% GFAP and 0.05 ± 0.02% O4 expressing cells were present in the cultures, underlining culture purity and suitability of the generated neuronal cell cultures (Fig. 1b, $N = 8$, $n = 3$, number of counted cells per $n \geq 176$). To further characterize the neuronal cell cultures, RT-

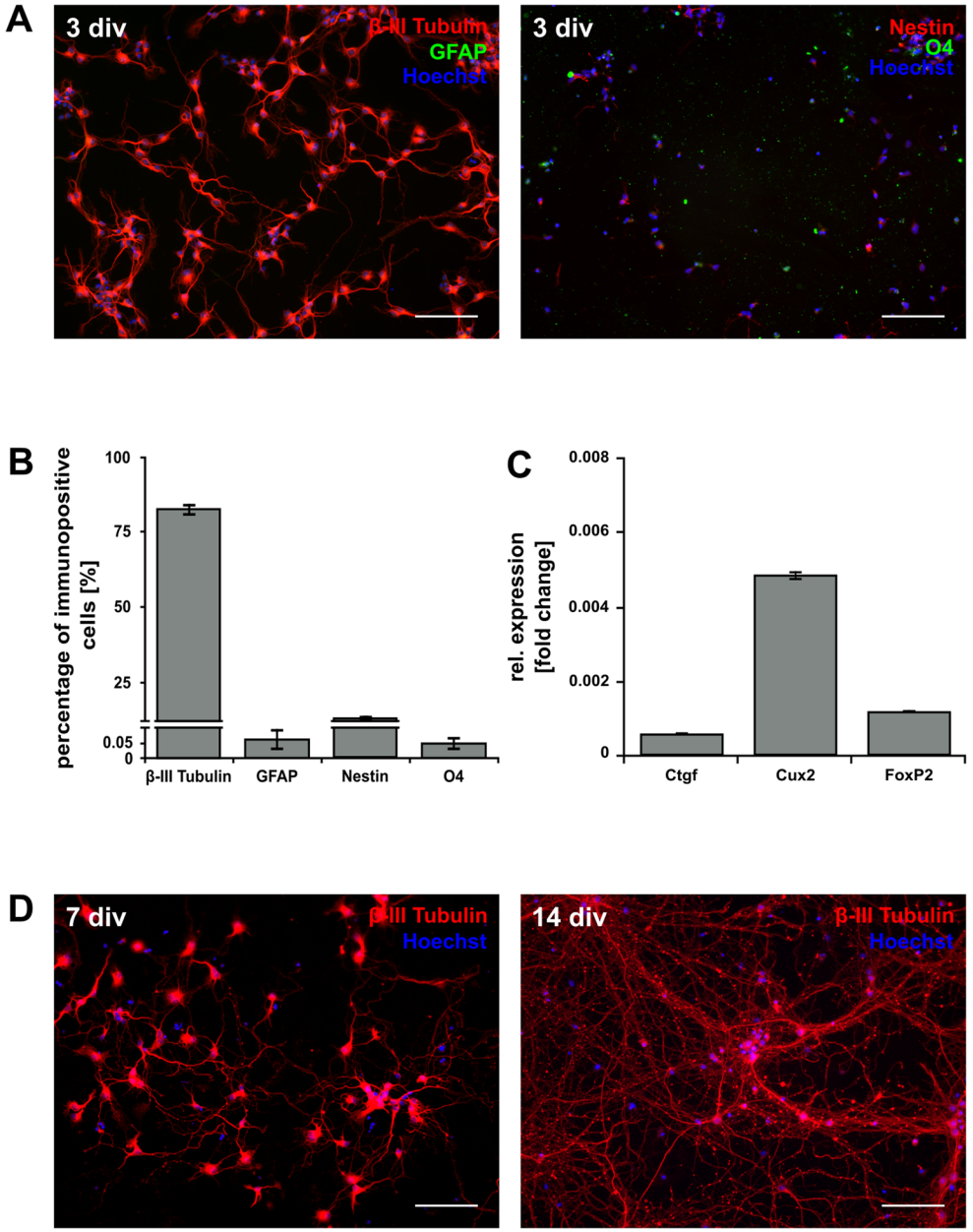



**Fig. 1** Establishment of a pure neuronal culture system from embryonic cortical tissue. **a** Representative photomicrographs of acutely dissociated cortical cells from embryonic mice after 3 days of in vitro cultivation, marked with immunological reagents detecting cell-type-specific epitopes of class III β-tubulin in neurons, GFAP in astrocytes, O4 in oligodendrocytes, and neural stem cell-associated nestin. The scale bar represents 50 μm. **b** Statistical evaluation of cell-specific epitope distribution within the established neuronal culture system. Evaluation is based upon eight independent approaches ($N = 8$, $n \geq 176$). **c** Respective gene marker expression averaged against the three housekeeping genes *18s RNA*, *cyclophilin*, and *β-actin*. RT-qPCR analysis of different layer-specific gene markers based upon four independent approaches ($N = 4$, $n = 3$). Here, *Ctgf* has been chosen for indicating early born subplate associated neurons, *Cux2* for identifying upper layer neurons, and *FoxP2* as a marker for deeper layers V and VI neurons. **d** Representative photomicrographs of neuronal cultures after 7 and 14 days of cultivation, highlighting the durability and purity of established neuronal cultures using immunocytochemical detection of neuron-specific class III β-tubulin and astrocyte-related GFAP epitopes. Scale bar represents 50 μm

qPCR analysis with cDNA templates from whole RNA lysates (3 days in vitro) was carried out (Fig. 1c, RT-qPCR analysis based on 4 independent approaches ($N = 4$, $n = 3$)). Previous studies reported expression patterns of specific genes during corticogenesis, giving the opportunity to identify different subtypes of neurons associated with specific cortical layers [32]. Hence, primers for amplification of layer restricted marker genes were generated to specify neuronal subtypes. During development of the mammalian cortex, upper-layer neurons are generated later than projection neurons of layers V and VI that exhibit characteristics of pyramidal neurons of a primitive cortex [32]. Therefore, primer pairs for amplifying

the cut like homeobox 2 (*Cux2*) transcription factor (identifying upper layer neurons), forkhead-box-protein P2 (*FoxP2*) transcription factor (for deeper layers V and VI neurons), as well as for connective tissue growth factor (*Ctgf*) (indicating early born subplate-associated neurons), were used for analysis. In this approach, cultivated neurons expressed foremost *Cux2* transcription factor, identifying them as upper layer neurons that populate layers II/III–IV (Fig. 1a). Other markers such as forkhead-box-2 (*FoxP2*) transcription factor, or *Ctgf* were less prominently expressed by the cultivated neurons.

## Introduction of VSWRAPTA Peptide to Neuronal Cultures Enhances Neuronal Network Complexity

Neuronal network formation is a highly regulated process, influenced by different cues of the extracellular matrix. In this context, Tnc represents a key molecule that is known to mediate neurite outgrowth precisely through specific domains [7, 8, 16–18]. Recently, our laboratory has reported the novel Tnc-derived VSWRAPTA peptide that affects significantly the migration behavior of different tumorigenic cell lines. Based on the location of the peptide sequence in proximity of domains that are known to affect neurite outgrowth, we were encouraged to test its potential to affect neurite formation in the embryonic neuronal culture system.

As described previously [19], we applied the VSWRAPTA peptide in soluble form to the cortical neuron cultures and assessed the necessary range of concentrations for proper peptide binding to putative receptors by comparing published data [19, 33, 34]. When applied to our cultures, neuritogenesis was significantly increased, saturating at a concentration of 50 μg/ml (representing a molarity of about 56 mM). While control cultures exhibited a specific neurite length after 3 days of cultivation, soluble addition of 10 μg/ml VSWRAPTA peptide led to an $1.13 \pm 0.06$-fold increase ($p > 0.05$, $N = 8$, $n = 3$, average number of counted cells per $n \geq 46$) when compared with untreated control cultures (Fig. 2a, c). Elevating the peptide concentration to 50 and 100 μg/ml, neurite lengths were promoted by factors of $1.34 \pm 0.05$ ($p < 0.001$, $N = 8$, $n = 3$, average number of counted cells per $n \geq 46$) and $1.29 \pm 0.04$ ($p < 0.01$, $N = 8$, $n = 3$, average number of counted cells per $n \geq 46$), respectively. To further provide evidence for sequence specificity, a scrambled version of the VSWRAPTA peptide was synthesized ($VAWTSPRA_{Scram}$). In doing so, we used the same composition of amino acids than in our native peptide, rather than an arbitrary amino acid mixture. Thereby, we compared the Tnc-derived peptide with a control peptide of conserved stoichiometry and charge. Amino acid exchanges were operated at four different positions. This choice was inspired by an earlier study reporting that the inversion of the classical RGD sequence at one position to RDG was sufficient to annihilate effects on cell adhesion and migration [35]. We reckon therefore that our strategy warrants a stringent examination of the relevance of the native amino acid sequence. The scrambled peptide ($VAWTSPRA_{Scram}$) was added at a concentration of 50 μg/ml, representing the most efficient concentration of the native Tnc peptide, and subsequently compared with the latter. The scrambled Tnc peptide did not promote neurite outgrowth to the same extent when compared with the correctly aligned sequence (Fig. 2b, d). Indeed, neurons stimulated by the native VSWRAPTA peptide displayed a clearly enhanced neurite outgrowth compared with the cultures under the influence of $VAWTSPRA_{Scram}$, underlining the specificity of this conserved amino acid sequence (Fig. 2d). These results are strongly indicative of a neurite-promoting effect of the VSWRAPTA sequence within the 6th constitutively expressed (corresponding to the third last) FNIII domain of the Tnc molecule.

## VSWRAPTA Promotes Neurite Branching of Cortical Neurons by Transcellular Activation of the Focal Adhesion Kinase FAK and Downstream ERK1/2 Signaling Pathway

In order to study the neurite-promoting effect of the VSWRAPTA peptide in more detail, neuronal morphology was investigated. Therefore, neurite branching and length measurements of single neurons were carried out by manual analysis using the NeuronJ [26] plugin for ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA). The analyses revealed that upon treatment with Tnc peptide, the branching behavior was significantly affected when compared with neurons in cultures receiving the scrambled variant or lacking peptide administration (Fig. 3a, b, d). In particular, the number of primary, secondary, and tertiary neurite branches appeared to be higher in comparison with control and scrambled peptide-supplemented cultures. Interestingly, the lengths of single branches of individual neurites remained unaffected (Fig. 3c). Thus, treatment with VSWRAPTA peptide does not foster the elongation of neurites per se but stimulates the branching and thereby the number of branch points (Fig. 3d) as well as the summed fiber length per neuron (Fig. 3e).

To provide further information about the VSWRAPTA sequence in the context of intracellular signal transduction, we additionally performed Western blot and RT-qPCR studies for specific marker proteins and molecules that are known to be involved in neurite outgrowth and branching. As one of the most ubiquitously activated signaling pathway involved in many different cellular processes, the FAK-triggered extracellular receptor kinase (ERK1/2)-mediated pathway plays a crucial role in neurite outgrowth events [36, 37]. When applied to neuron cultures, 50 μg/ml of VSWRAPTA peptide induced phosphorylation of the focal adhesion kinase. An initial increase of phosphorylation became apparent already after 10 min and achieved enhanced intensity and statistical

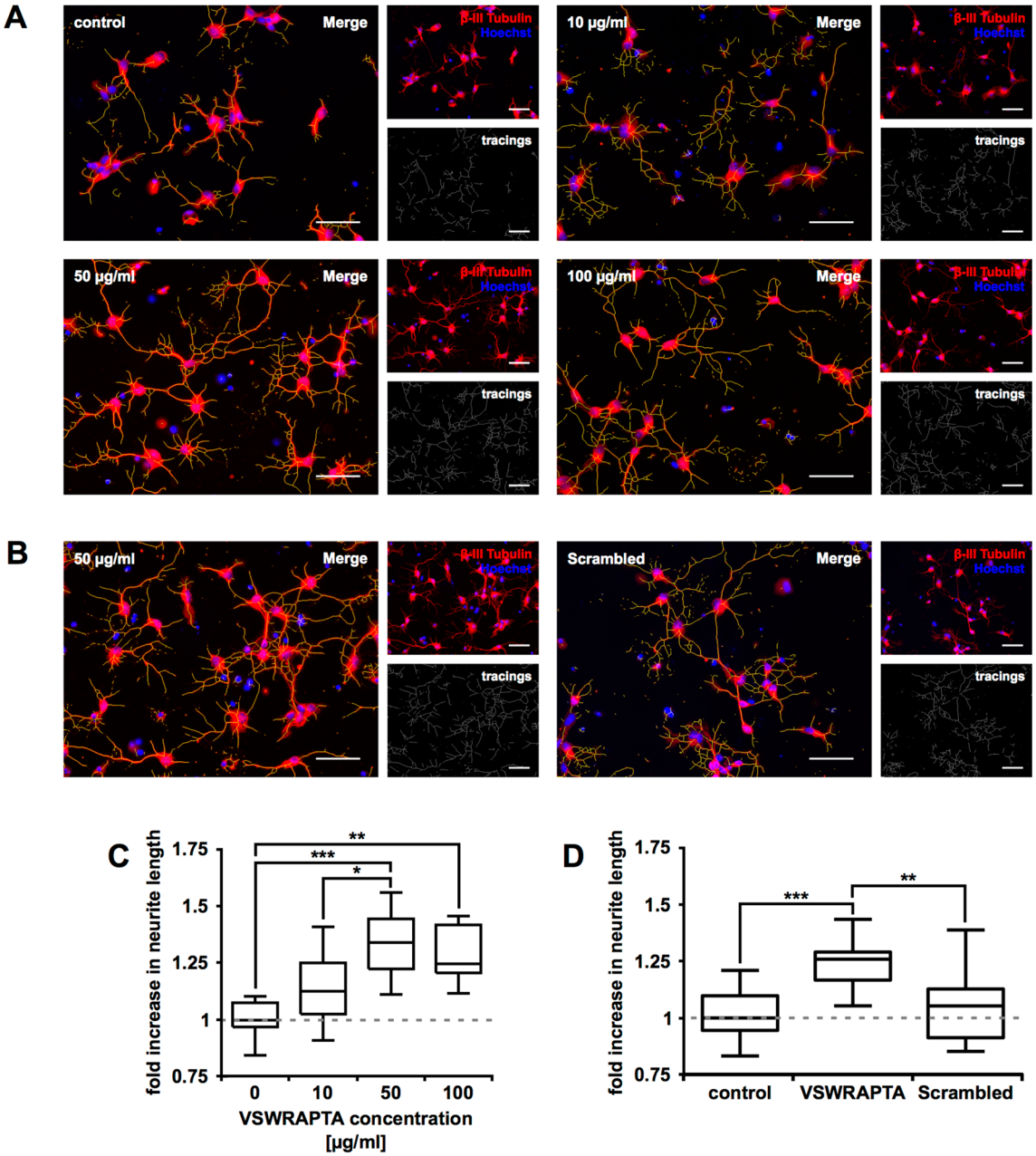



**Fig. 2** Analysis of neurite outgrowth after 3 days of cultivation. **a** Representative photomicrographs of neuronal cultures after 3 days of in vitro cultivation under control conditions or supplemented with the Tnc-derived VSWRAPTA peptide at concentrations of 10, 50, and 100 µg/ml. Neuronal tracing analysis was performed using ImageJ analysis software with the plugin extension Neurite Tracer [25]. Scale bar represents 50 µm. **b** Photomicrographs of neuronal cultures supplied with VSWRAPTA or the corresponding scrambled peptide; both cultures were supplemented with 50 µg/ml of respective peptide. Scale bar represents 50 µm. **c** Statistical evaluation and comparison of average neurite lengths of treated and untreated control cultures. Neurite outgrowth was significantly promoted with increasing VSWRAPTA peptide concentrations saturating at 50 µg/ml. Values are based on eight independent approaches ($N = 8$, $n \geq 46$). Significance was assessed by one-way analysis of variance (ANOVA) with the Scheffé procedure for post hoc test. Data are indicated as mean ± standard error of mean (SEM) with $p$ values of $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$. **d** Statistical evaluation and comparison of average neurite lengths of cultures exposed to VSWRAPTA peptide, scrambled peptide ($VAWTSPRA_{Scram}$) (both 50 µg/ml), or no additional peptide (control). Analysis based upon eight independent approaches ($N = 8$, $n \geq 46$), with significance-level assessment by one-way analysis of variance (ANOVA) with the Tukey procedure for post hoc test. Data are indicated as mean ± SEM with $p$ values of $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$. Note that cultures supplemented with soluble Tnc peptide displayed significantly boosted neurite outgrowth in direct comparison with the scrambled peptide version and control

significance after 120 min (Fig. 4b). In contrast, similar amounts of $VAWTSPRA_{Scram}$ did not lead to activation of FAK (Fig. 4a, b). A comparable kinetic of activation of FAK has been reported with regard to the adhesion and invasion of

pancreatic cancer cells [38]. In addition, phosphorylation of extracellular signal-regulated kinase (ERK1/2) was triggered when compared with untreated control cultures (Fig. 4c). Although the scrambled Tnc peptide also induced phosphorylation of ERK1/2 in neuron cultures, the biomimetic VSWRAPTA peptide displayed a much stronger and more prolonged response (Fig. 4c). This might reflect a residual activity of the scrambled peptide due to the conserved stoichiometry that we applied to increase the stringency of the control (see above). Also, it is worth considering that in particular the ERK1/2 signaling pathway is sensitive to any kind of physical, chemical, and osmotic stress that might be caused in the course of peptide administration [39].

Beyond the impact on signal transduction, elevated expression profiles of specific neurite outgrowth associated genes could be identified upon introduction of 50 μg/ml VSWRAPTA (Fig. 4d–f). As previously reported, Tnc-mediated neurite outgrowth is associated with contactin binding [8, 40]. In this perspective, we investigated whether mRNA transcripts of putative Tnc receptors were modified by peptide treatment. For example, Contactin-3 (*Cntn3*) which has been found up-regulated in the context of neurite extension [41] was significantly increased in cultures supplemented with both, the correctly aligned VSWRAPTA peptide and its scrambled version $VAWTSPRA_{Scram}$ (Fig. 4f). Interestingly, the neurite branching related transcription factor forkhead-box-protein P2 (*FoxP2*) [42] was significantly downregulated in cultures exposed to the scrambled Tnc peptide, but slightly upregulated in cultures supplemented with the native Tnc sequence VSWRAPTA (Fig. 4e), in agreement with the analysis of branch points in treated cultures (Fig. 3d).

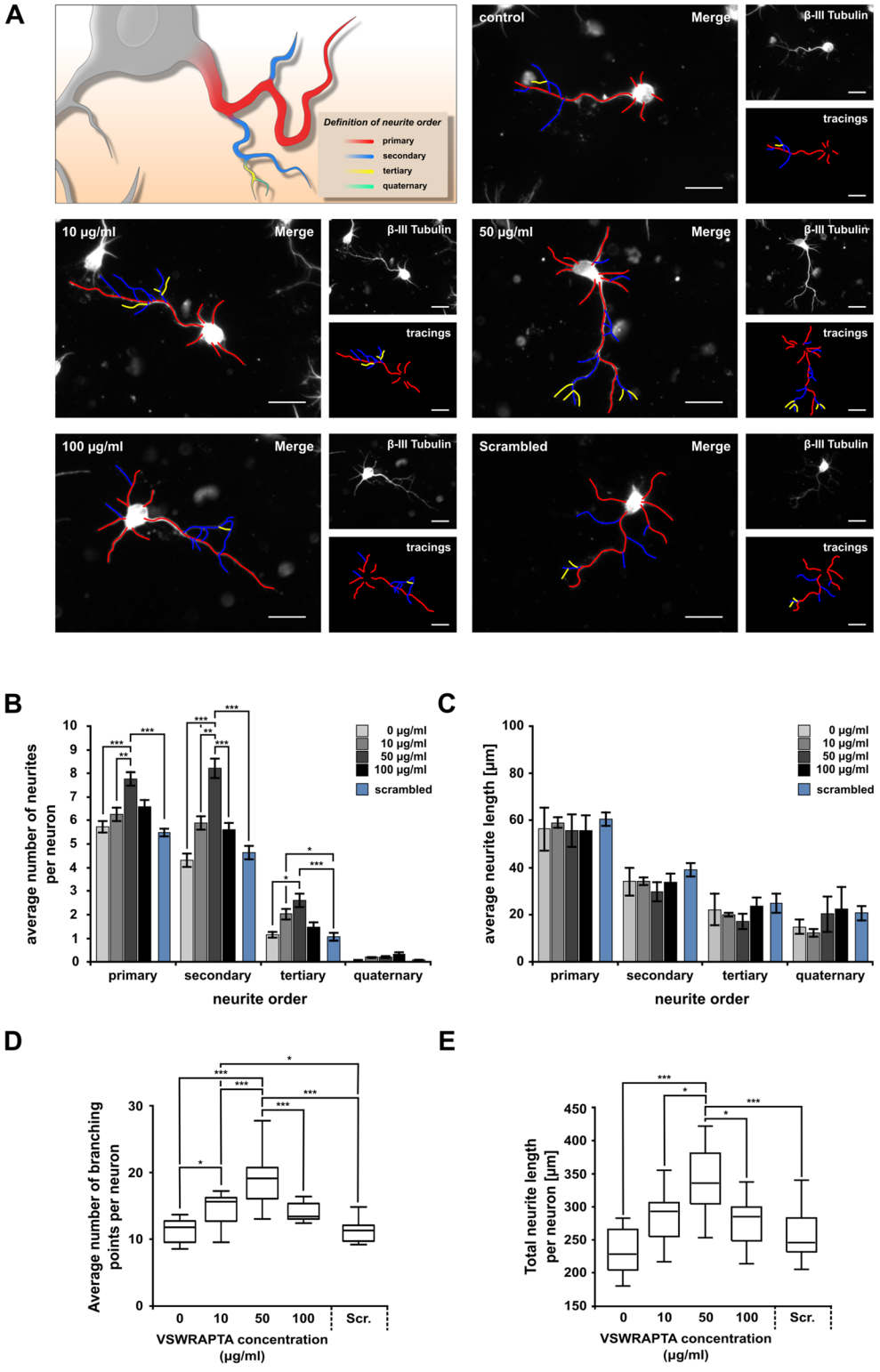
Several integrin receptors have been proposed for Tnc that bind, however, to domains remote from the one harboring the VSWRAPTA peptide sequence [17, 43]. Integrin clustering can result as a feedback between integrin activation and binding, an auto-regulatory mechanism that triggers the expression of integrin subunits [44]. This feedback loop may be launched by the bioactive peptide. To further address the question whether integrin receptors are involved in response to the peptides assessed in this study, mRNA expression levels of several candidate integrin subunits were analyzed. While β1-integrin expression levels appeared to be elevated in the presence of both the native or scrambled peptide sequence (Fig. 4d), only cultures treated with the VSWRAPTA peptide displayed higher expression levels of the neuronal differentiation promoting αV integrin subunit [45, 46]. The neurite outgrowth promoting subunit α7 [17] and the differentiation driving β3 integrin subunit [45, 46] appeared slightly elevated in direct comparison (Fig. 4d). Taken together, the results suggest that the VSWRAPTA peptide stimulates the branching and overall length of neurites of embryonic cortical neurons in culture (Fig. 5).

**Fig. 3** Analysis of neurite formation and branching on single cell level after 3 days of cultivation. **a** Schematic illustration of neurite classification and representative photomicrographs of individual neurons cultured in the presence or absence of the Tnc-derived VSWRAPTA or the corresponding scrambled peptide at indicated concentrations. Neurite branching and length were manually measured after 3 days of in vitro cultivation, using ImageJ analysis software and plugin extension NeuronJ [26]. Scale bar represents 20 μm. **b**–**e** Statistical evaluation and comparison of individual neuronal characteristics of cultures shown in (**a**). In total, 60 individual neurons were analyzed per condition ($N = 12$, $n = 5$), with significance levels attested by one-way analysis of variance (ANOVA) with Scheffé procedure for post hoc testing. Data are indicated as mean ± standard error of mean (SEM) with $p$ values of $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$. **b** Statistical analysis of neurite branching. Branching was significantly increased upon treatment with the correctly aligned Tnc peptide in a dose-dependent manner saturating at concentrations of 50 μg/ml. **c** Statistical evaluation and comparison of individual neurite lengths, compared within the different branching categories (see (**a**)). Here, the average neurite lengths did not differ within compared groups. **d** Statistical evaluation of the average branching point number of neurons under different conditions. The average branching point number saturates at a concentration of 50 μg/ml of native VSWRAPTA peptide, whereas cultures under influence of the scrambled peptide did not show changes in branching point occurrence. **e** Statistical assessment of the average total neurite lengths of neurons under different conditions. Again, highest values were achieved with native VSWRAPTA peptide concentrations of 50 μg/ml

## Discussion

Tenascin-C is an important constituent of the ECM of the developing CNS. There, it is associated with neural stem and progenitor cells and involved in axon growth and guidance [3, 5, 14]. Single subunits of the Tnc multimer consist of EGF-type repeats, FNIII domains, and sequences homologous to fibrinogen-β [47, 48]. An alternative splice site is known between the 5th and the 6th FNIII repeats of the basic structure, and a large number of isoforms based on the combinatorial variation of additional six alternatively spliced FNIII repeats has been documented in the mouse (Fig. 6) [2, 9, 10]. Investigations of the variable splice variants revealed that the combinations of the FNIII domain D with the 6th constitutively expressed FNIII domain (TNfnD6-Fc) promoted neurite outgrowth of embryonic hippocampal neurons [7, 8]. More in-depth analyses proposed the specific sequence of eight consecutive amino acids VFDNFVLK within the TNfnD domain of alternatively spliced FNIII repeats as responsible for the neurite outgrowth-promoting effect of Tnc [16]. The peptide by itself promotes neurite extension in neuronal cultures in vitro and systematic variation of the sequence indicated that the amino acid pairs FD and FV therein are crucial for its functionality [16].

In the present study, we examined the Tnc-derived peptide motif VSWRAPTA (Fig. 6) that enhances the migration rate of CNS- and mesenchyme-derived tumor cell lines [19], as well as its scrambled isoform variant $VAWTSPRA_{Scram}$ with regard to putative neurite outgrowth-promoting properties. To




this end, we initially established a protocol for the generation of a pure cortical neuronal culture from embryonic mouse CNS. In this in vitro model, neurons can be cultivated for up to 14 days without additional feeder layer. In approaches using the co-culture strategy, glia cells prominently support the maintenance and affect the morphology as well as the fate of neurons during specific developmental stages and even later in adulthood [50–53]. In order to rule out any secondary effects caused by glial cells that might as well be influenced by peptide addition, pure cultures of mouse embryonic E12.5 cortical hemispheres neuronal cultures were used. Neurons at specific developmental stages express specific gene

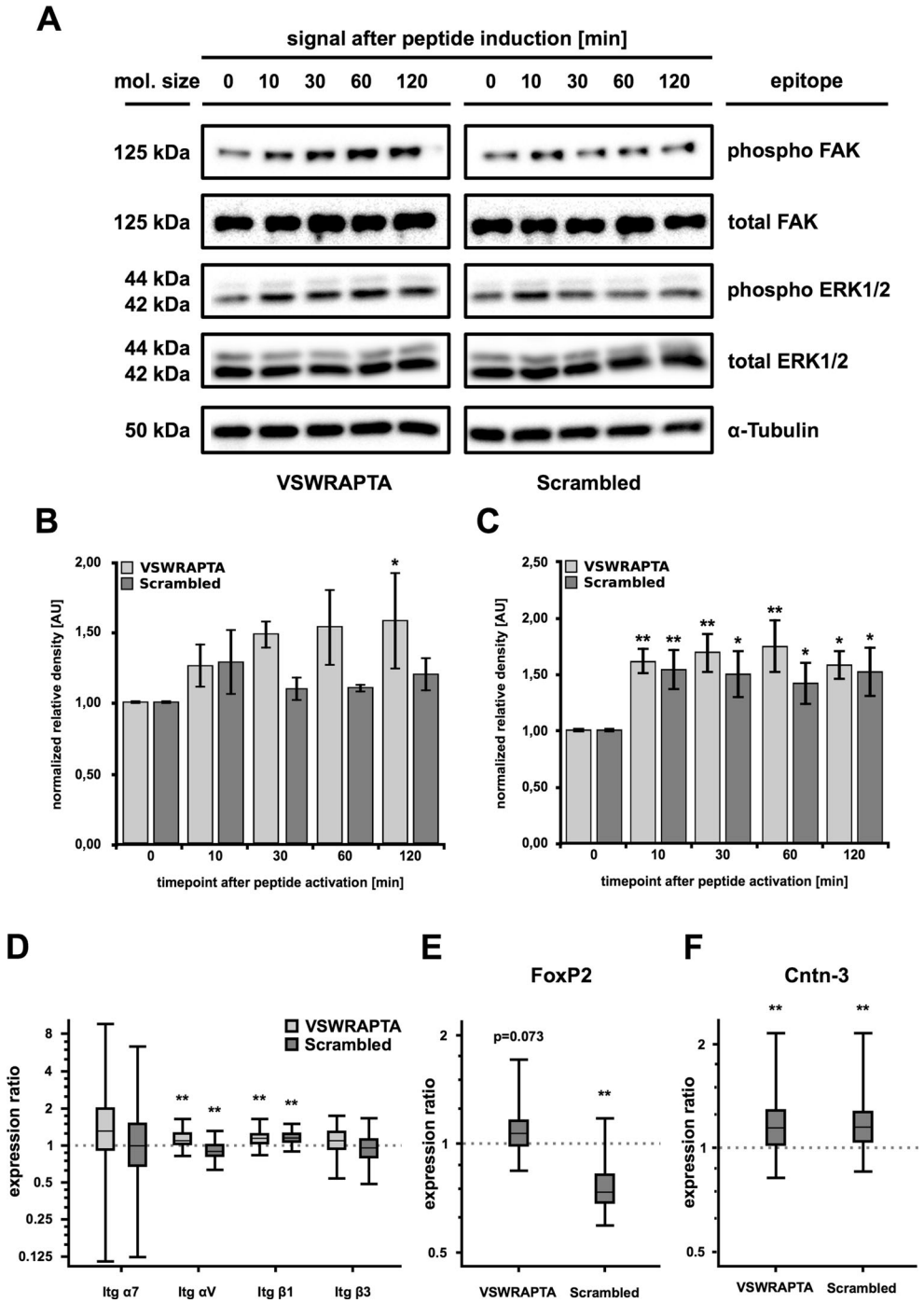



**Fig. 4** Analysis of VSWRAPTA peptide induced intracellular signaling. **a** Representative photomicrographs of performed Western blot analyses for focal adhesion kinase (FAK), tyrosine phosphorylated FAK, extracellular receptor kinase (ERK1/2), phosphorylated ERK1/2, and α-Tubulin. Here, different time points after peptide administration are displayed (VSWRAPTA, 50 μg/ml; scrambled peptide, 50 μg/ml). **b** Densitometric evaluation of phosphorylated FAK of cultures subjected to native and scrambled peptide (both 50 μg/ml). Three independent experiments were performed ($N = 3$). Significance was assessed by one-way analysis of variance (ANOVA) with the Bonferroni procedure for post hoc tests. Data are indicated as mean ± standard error of mean (SEM) with $p$ values of $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$). **c** Densitometric analysis of phosphorylated ERK1/2 of cultures subjected to native and scrambled peptide (both 50 μg/ml), based on four independent experiments ($N = 4$). Significance was determined by ANOVA and the Bonferroni correction for the post hoc test. Data are given as mean ± SEM with $p$ values of $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$). **d**–**f** RT-qPCR analysis of neurite outgrowth-associated genes based on cDNA transcripts of mRNA lysates, isolated from neuronal cultures exposed to VSWRAPTA peptide (50 μg/ml), scrambled peptide, VAWTSPRA (50 μg/ml), or untreated control cultures. Isolation of mRNA was performed after 3 days of cultivation. Statistical evaluation and comparison of individual gene marker expression for the different treatment conditions is based on four, five, and three independent experiments for (**d**) ($N = 4$), (**e**) ($N = 5$), and (**f**) ($N = 3$), respectively. Peptides were applied at concentrations of 50 μg/ml. Significance was determined by two-way ANOVA, using the Scheffé procedure for post hoc tests. Data are indicated as mean ± SEM with $p$ values of $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$

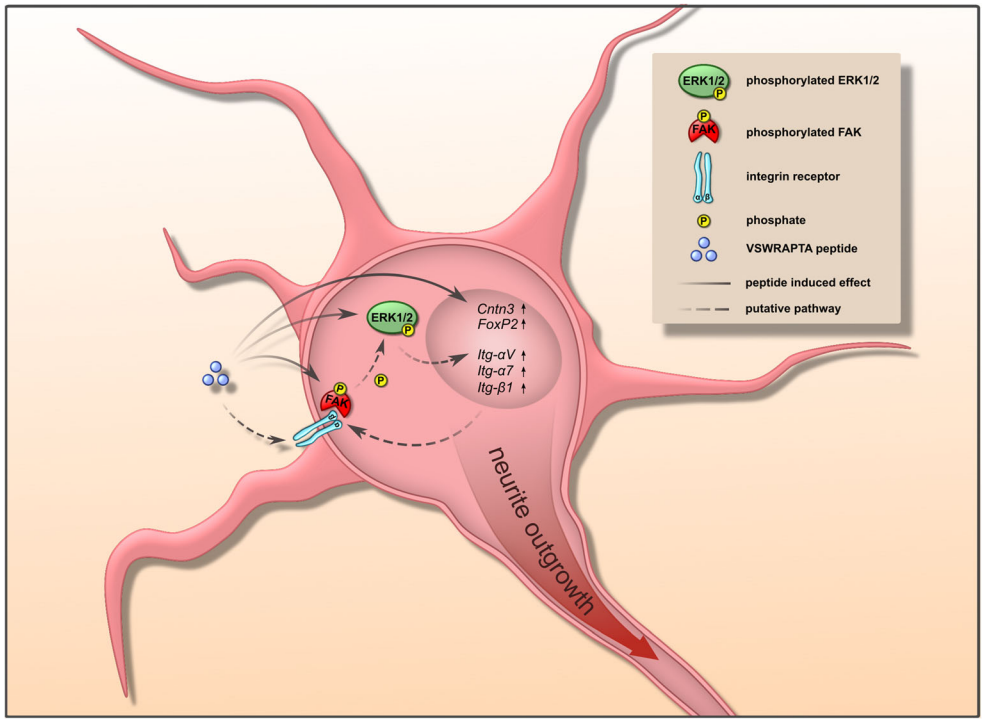



**Fig. 5** Schematic illustration of the proposed effect of VSWRAPTA peptide administration to primary neuron cultures. Soluble administration of the VSWRAPTA peptide induces the phosphorylation of the intracellular FAK and ERK1/2 proteins, which are known to facilitate neurite outgrowth and neuronal maturation. In parallel, the neurite outgrowth associated genes CNTN3 and FoxP2 are upregulated on the mRNA level. Thus, we propose that the Tnc-derived peptide interacts with receptors that initiate the phosphorylation of FAK and subsequently ERK1/2. Phosphorylated ERK1/2 may serve as downstream signal, inducing the expression of neurite outgrowth associated genes such as *Cntn3* and *FoxP2*, which in turn could promote neurite outgrowth

markers, which can be used to characterize and determine the origin of isolated neurons [32]. Along these lines, the RT-qPCR analysis of the cortical cultures revealed that the neurons predominantly expressed the layer II/III-IV-associated gene marker *Cux2*, identifying them as upper-layer neurons. At later developmental stages, this subgroup of neurons will progressively differentiate into callosal projection neurons in vivo [32]. Usually, this type of neuron is generated at around E13.5–E15.5. At around E15.5, deeper-layer neurons emerge, expressing characteristically the transcription factor *FoxP2* [54]. Yet, cells isolated from dissected cortical tissue at embryonic stage E12.5 may give rise to later-born neuronal types upon additional in vitro cultivation, which would explain the observation of more mature markers [55]. Morphologically, this type represents medium-sized pyramidal neurons, with characteristic axonal projections extending across the corpus callosum to different brain areas [32]. Thus, the established neuronal culture provides a good basis for further analyses regarding the neurite-promoting effect of the Tnc peptide.

In this culture model, the addition of the synthetic Tnc-derived peptide VSWRAPTA indeed promoted neurite outgrowth in a dose-dependent manner. While neurons exhibited a continuous neurite outgrowth throughout the monitored cultivation period, the soluble peptide increased the numbers of neurites significantly when supplemented at a concentration of 50 μg/ml. This prominent effect was controlled by introduction of the scrambled version $VAWTSPRA_{Scram}$ that does not naturally occur in the mouse CNS. When used at the saturating concentration of 50 μg/ml, the scrambled peptide proved clearly less efficient than the integral VSWRAPTA test peptide, consistent with a specific efficacy of the latter. Interestingly, the neurite outgrowth-promoting VFDNFVLK peptide has previously been reported within the TNfnD domain of Tnc [7, 16, 17], with a location different from the VSWRAPTA peptide (Fig. 6). On the other hand, the investigation of the neurite outgrowth-promoting domain using recombinant proteins corresponding to different splice variants of Tnc suggested the existence of a neurite growth promoting site in TNfn6 of the constitutive structure [8]. Therefore, we performed more in-depth analysis focusing on neurite branching where neurons were supplemented with synthetic peptides. The analysis of individual neurons clearly showed increased numbers of neurites, augmented number of branch points and, consequently, of secondary neurites. Their number doubled in comparison with the situation in control and scrambled peptide treated cultures. Hence, neurite branching seemed to be more affected than the individual fiber length. This interpretation was further supported when average neurite lengths of neurites within specific branching orders (primary, secondary, and tertiary of quaternary neurites) were

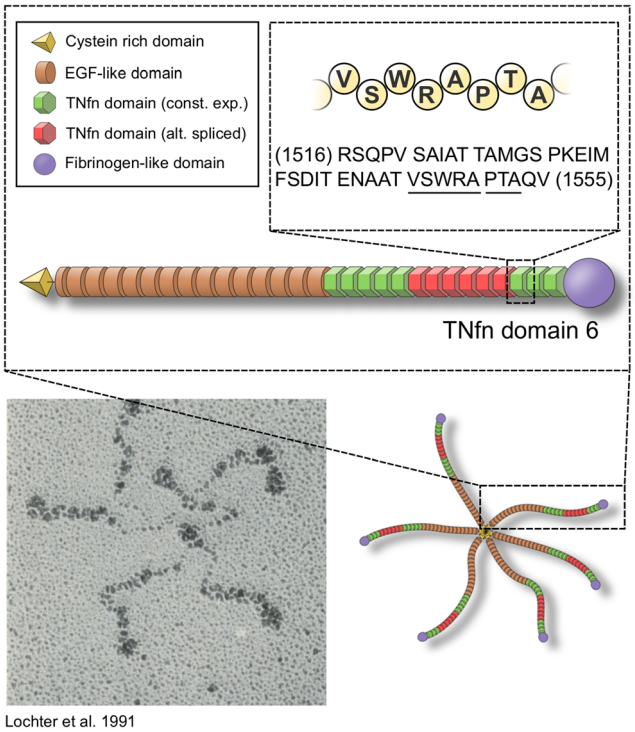



**Fig. 6** Location of the VSWRAPTA motif within the Tnc molecule. The extracellular matrix molecule tenascin-C represents a multimodular hexameric glycoprotein. The bioactive motif with its neurite outgrowth-affecting characteristics is located within the sixth constitutively expressed tenascin fibronectin-like III (TNfnIII) domain. The exact location of the motif is illustrated in the mouse Tnc molecule amino acid sequence. The electron micrograph showing a rotary shadowed preparation of Tnc purified from mouse CNS is republished with permission of Journal of Cell Biology, taken from [49]; permission conveyed through Copyright Clearance Center, Inc.

analyzed and compared. Interestingly, no differences in average length could be determined, whether neurons were supplemented with the native or with the scrambled TNfn6-derived peptide. This supports the interpretation that the Tnc VSWRAPTA sequence fosters the number of neurite branch points without, however, modifying the speed of neurite extension. As a consequence, the number of fibers per neuron and their total length were considerably increased. In conclusion, it appears that the VSWRAPTA peptide selectively stimulates sprouting and does not affect growth rates of fibers. This observation clearly differs from previous studies that characterized an active peptide sequence of the alternatively spliced TNfnD domain close to the alternative splice site [16–18]. These authors emphasized that the overall lengths of the longest neurites were considerably expanded by treatment with the VFDNFVLK peptide.

Neurite outgrowth and branching are events that rely on manifold external stimuli, relevant receptors and downstream signaling cascades that impact the cytoskeleton [56]. Therefore, we sought to clarify potential intracellular signaling mechanisms triggered by the addition of peptides. Furthermore, as Tnc is known to interact with a variety of integrin receptors [43], we scanned the cortical neurons for the expression of integrin receptor subunits. Beyond their role as Tnc receptors, integrins are also known to contribute to neuronal differentiation and neurite outgrowth [17, 45]. Activation of integrins can result in auto-regulatory mechanisms that stimulate the elevated expression of respective integrin subunits, which can be revealed by qPCR analysis [44]. Interestingly, in that comparable with the earlier report concerning the VFDNFVLK peptide of TNfnD, the TNfn6-derived peptide VSWRAPTA studied in our laboratory enhanced the expression of β1 and αV integrin expression. Furthermore, the mRNA levels of the neurite length stimulating Itgα7 [17] and the differentiation-related Itgβ3 integrin subunit [45, 46] seemed slightly elevated in the presence of the VSWRAPTA peptide. Interestingly, Itg$\alpha_7\beta_1$ and/or Itg$\alpha_V\beta_3$ expressed by neurons result in enhanced neurite outgrowth [17] and neuronal differentiation [45]. It is conceivable that the Tnc-derived bioactive sequence motif VSWRAPTA is recognized by distinct integrin subunits. The identification and characterization of the receptor engaged represents a rewarding objective for future studies.

In order to gain insight into the signal transduction mechanism involved, we next investigated the activation of the FAK and the ERK1/2 by exposure to the peptide. Earlier studies have highlighted the relation between ERK1/2 signaling and neurite outgrowth [57, 58]. In addition, a direct link between integrin activation and ERK1/2 phosphorylation has been demonstrated in the context of osteogenic differentiation [59]. In our study, the addition of VSWRAPTA to the neurons selectively induced the phosphorylation of focal adhesion kinase, whereas both the correctly aligned and the scrambled peptide elevated the phosphorylation of ERK1/2. The combined activation of FAK and ERK1/2 might be relevant for the increased branching observed in our approach [60, 61], but this has to be clarified in further investigations. Finally, examination of several target genes that have been implicated in the process of neurite outgrowth completed our analysis [41, 42, 62]. Tnc is specifically regulated during embryonic development and its various functions with regard to cellular differentiation are a result of cell surface receptor relayed intracellular signaling [63–65]. Contactin-3 (*Cntn3* or *Big-1*), a putative regulator of neurite outgrowth in neurons [41, 62] appeared significantly upregulated in the presence of VSWRAPTA. *Cntn3* is involved in adhesion and migration processes and guides neurite outgrowth in vitro [41, 62]. Although no evidence of Cntn3 and Tnc interaction has been reported so far, this assumption could provide an explanation for the results, as Tnc has been proven to interact with the related contactin-1 [8]. Interestingly, the scrambled peptide diminished *FoxP2* transcription factor expression. *FoxP2* regulates a set of genes that are associated with controlling the length and branching of neuronal projections [42].

In summary, we have established that the recently introduced peptide VSWRAPTA that is derived from the 6th constitutively expressed FNIII domain of Tnc promotes neurite branching when added in soluble form to embryonic cortical neuron cultures. The peptide stimulates the activation and phosphorylation of FAK and ERK1/2, and it modulates the expression of neurite outgrowth-associated genes.

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