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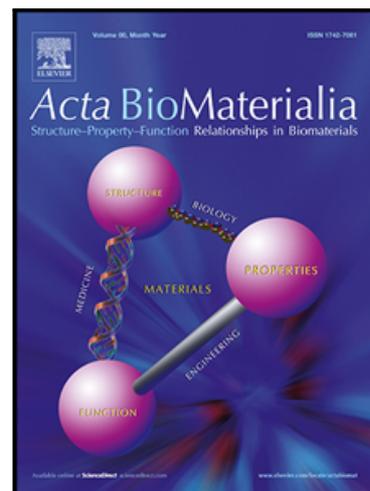
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Biomaterial based strategies to reconstruct the nigrostriatal pathway in organotypic slice co-cultures

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BU: Investigation, Methodology, Visualization, Formal analysis, Writing - original draft, Writing - review & editing. **JK:** Methodology, Writing - review & editing. **BMF:** Visualization. **DE, CW and JB:** Resources. **KRL:** Resources, Writing - review & editing. **JE and AP:** Supervision. **ML:** Resources, Methodology, Writing - review & editing. **BN:** Resources, Supervision, Writing - review & editing. **CH:** Conceptualization, Methodology, Supervision, Project administration, Resources, Writing - original draft, Writing - review & editing, Funding acquisition

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Statement of significance: The degeneration of the nigrostriatal pathway is a main pathology in Parkinson's disease (PD). Protective or repair strategies for the nigrostriatal pathway can employ various biomaterials and therapeutic molecules loaded into these materials. In this study, we use an *ex vivo* model of organotypic brain slices by culturing together the brain regions involved in the pathway. By using this model, we evaluate several biomaterials for the repair of the nigrostriatal pathway and the effects of incorporating glial cell line-derived neurotrophic factor (GDNF), a potent neurotrophic factor. The results of this study provide a general overview of the advantages and disadvantages of different approaches for future regenerative strategies and tissue engineering applications.

Graphical abstract

Abstract

Protection or repair of the nigrostriatal pathway represents a principal disease-modifying therapeutic strategy for Parkinson's disease (PD). Glial cell line-derived neurotrophic factor (GDNF) holds great therapeutic potential for PD, but its efficacious delivery remains difficult. The aim of this study was to evaluate the potential of different biomaterials (hydrogels, microspheres, cryogels and microcontact printed surfaces) for reconstructing the nigrostriatal pathway in organotypic co-culture of ventral mesencephalon and dorsal striatum. The biomaterials (either alone or loaded with GDNF) were locally applied onto the brain co-slices and fiber growth between the co-slices was evaluated after three weeks in culture based on staining for tyrosine hydroxylase (TH). Collagen hydrogels loaded with GDNF slightly promoted the TH⁺ nerve fiber growth towards the dorsal striatum, while GDNF loaded microspheres embedded within the hydrogels did not provide an improvement. Cryogels alone or loaded with GDNF also enhanced TH⁺ fiber growth. Lines of GDNF immobilized onto the membrane inserts via microcontact printing also significantly improved TH⁺ fiber growth. In conclusion, this study shows that various biomaterials and tissue engineering techniques can be employed to regenerate the nigrostriatal pathway in organotypic brain slices. This comparison of techniques highlights the relative merits of different technologies that researchers can use/develop for neuronal regeneration strategies.

Key words: Nigrostriatal pathway regeneration, hydrogel, cryogel, microcontact printing, organotypic brain slices, GDNF

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1. Introduction

The nigrostriatal pathway, where substantia nigra (SN) dopaminergic neurons innervate the dorsal striatum (doSt), is one of the major dopaminergic pathways of the brain. This pathway is involved in motor function and its disruption causes disorders such as Parkinson's disease (PD). The main pathology of PD is the loss of dopaminergic neurons in the SN, which reduces dopamine levels in its striatal target region [1]. Recent studies suggest that axonal degeneration is predominantly involved in the earlier stages of PD, much before the loss of dopaminergic cell bodies [2,3]. Many protective and regenerative strategies for PD therapies focus on protecting the dopaminergic neurons of the SN, but the innervation of these neurons in their target regions is of clear importance [4]. Protection or restoration of this pathway would go beyond current symptomatic therapeutics and provide a disease-modifying therapy that may halt or even reverse the disease progression [5].

Organotypic brain slices are important *ex vivo* models for studying PD. Brain slices have the advantages of preserving the three-dimensional architecture of the tissue and including all the cell types of the central nervous system. Organotypic brain slices constitute a bridge between primary cell cultures and *in vivo* animal experiments by allowing greater complexity in comparison to *in vitro* models, together with greater flexibility than the *in vivo* models [6]. Several organotypic models have been established in order to study the nigrostriatal degeneration and regeneration. Some of these studies focus on establishing sagittal organotypic slices, as these slices contain both SN and striatum [7–9]. However, it is challenging to preserve the nigrostriatal pathway during the slicing procedure in this model, which requires very thick slices (350-400 μm) [7,9] or a modification of the slicing procedure (e.g. changing the slicing angle) [8]. Co-slices of different regions have been established to explore the reinnervation capacity of dopaminergic neurons [10–12]. Indeed, dopaminergic neurons in co-slices showed a selective target-oriented fiber growth towards the striatum in these models [12].

Glial cell line-derived neurotrophic factor (GDNF) is a potent trophic factor for dopaminergic neurons of the midbrain and a promising candidate for improving nigrostriatal pathway regeneration. GDNF significantly enhances dopaminergic neurite growth in primary dopaminergic cell cultures [13]. In clinical trials GDNF was shown to increase neural sprouting in the dorsal striatum [14]. However, the site of administration of GDNF has critical importance, as it may hinder or even block the innervation of target regions and subsequent functional recovery [4]. Likewise, in organotypic co-slice models application of GDNF simply to the culture medium did not mediate a targeted axon growth and caused innervation of non-targeted regions such as cortex and hippocampus [12].

Targeting GDNF to specific brain regions may not only improve nerve fiber innervation but may also prevent off-target fiber growth. Biomaterials are good candidates to improve directed fiber innervation and may play an important role in providing GDNF-mediated neuroprotection and neuronal guidance [15]. Suitable biomaterials could exist in many forms such as hydrogels, microspheres, cryogels and GDNF-immobilized surfaces. Of these materials, collagen hydrogels have numerous advantages, such as being biocompatible and biodegradable, causing no toxicity and supporting axonal growth in central and peripheral nervous systems [16,17]. We have previously shown that collagen hydrogels crosslinked with polyethylene glycol (PEG) protect axotomized cholinergic neurons when loaded with nerve growth factor (NGF) [18] and provide neuroprotection in three different models of PD when loaded with GDNF [17]. These collagen hydrogels can provide a localized source of GDNF in target tissues to attract dopaminergic fiber growth.

Hollow microspheres have also been extensively investigated for sustained and targeted delivery of GDNF to the brain [19]. The small size of these spheres make them suitable for intra-striatal injection [20]. Microspheres made of poly(allylamine hydrochloride) (PAH) and dextran sulfate (DEX) functionalized with tannic acid (TA) show significant antioxidant properties and retain the bioactivity of encapsulated molecules [21]. Loading GDNF into such microspheres could prolong the release time and allow for high concentrations of the trophic factor to be released locally [19]. Furthermore, unlike the collagen hydrogels mentioned above, microspheres can be prepared and loaded in advance of the application.

Another class of biomaterial that can be prepared and dried prior to use are cryogelated hydrogels (cryogels). Although similar in chemical composition to their hydrogel counterpart, the sub-zero polymerization results in a mechanically robust, yet soft macroporous structure [22]. Unlike hydrogels that can lose their shape or mechanical stability in *ex vivo* cultures [23], cryogels exhibit shape memory properties [24] that are ideal for *ex vivo* applications [25]. Poly(ethylene glycol) diacrylate (PEGDA) based cryogels were successfully used in organotypic slice cultures of spinal cord and brain cortex for local application of lysophosphatidylcholine (LPC) to induce focal demyelination and create a focal multiple sclerosis model [25]. To the best of our knowledge, there are currently no published data on the use of cryogels for delivering trophic factors to dopaminergic neurons.

Another approach to stimulate guided neural fiber outgrowth is to immobilize GDNF on the surface that the fibers are growing along. Microcontact printing is a soft lithography technique that has been used to immobilize proteins onto surfaces [26-28]. Microcontact printing takes advantage of an elastomer stamp, commonly made of polydimethylsiloxane (PDMS), which adsorbs proteins from an "ink" solution and transfers these proteins to a

substrate with very high resolution. Microcontact printing has many advantages over alternative techniques. Firstly, most proteins can retain their biological activity following microcontact printing [27]. Secondly, it is compatible with methods such as immunohistochemistry. Lastly microcontact printing is simple, cheap, efficient, and reproducible [27,28]. Microcontact printing of proteins onto unconventional materials such as insert membranes could provide new opportunities for precise manipulation in *ex vivo* models and various other tissue engineering approaches.

In the present study, we used a well-established organotypic co-slice model of nigral dopamine neurons and their target regions. The aim of the study was to evaluate and compare various robust biomaterial approaches, such as collagen hydrogels, PAH/DEX-TA microspheres, cryogels and a microcontact printing technique for local application of GDNF to organotypic brain co-slices to re-establish the nigrostriatal pathway.

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2. Methods

2.1 Organotypic brain slice cultures

Organotypic brain slice co-cultures of the ventral mesencephalon (vMES) and dorsal striatum (doSt) together with the surrounding cortex (Ctx) were prepared based on previous work [17,29]. All animal experiments were approved by the Austrian Ministry of Science and Research and conformed to the Austrian guidelines on animal welfare and experimentation. Postnatal day 9-11 C57BL/6 mouse pups were rapidly decapitated and the brains were dissected under sterile conditions. Brains were glued (using Loctite 404) to the sample holder of a water-cooled vibratome (Leica VT1000A). Coronal slices with 150 μm thickness were cut in sterile preparation medium (50% MEM/HEPES (Gibco), 2mM NaHCO_3 (Merck), pH 7.2) from striatum and vMES areas. From each mouse, three slices were taken from the striatal region (that were divided from the middle into two equal pieces) and six slices were collected from the vMES region (see Fig.1A). These slices were cut in half, placed as co-slices of striatum and vMES half slices on additional membranes (Merck, HTTP02500) and put on membrane inserts (Millipore PICM03050) in a 6-well plate (Greiner) (see Fig. 1A). Slices were placed together for initial experiments with collagen hydrogels and had a gap in between for further experiments with collagen hydrogels, microspheres, cryogels and microcontact printing. The gap between the slices provided an area to clearly observe and visualize TH+ fiber growth without the background from the tissue. The co-slices were cultured at 37°C and 5% CO_2 with 1 mL of slice culture medium (50% MEM/HEPES (Gibco), 25% heat-inactivated horse serum (Gibco/Lifetech), 25% Hanks' solution (Gibco), 2 mM NaHCO_3 (Merck, Austria), 6.5 mg/mL glucose (Merck), 2 mM glutamine (Merck), pH 7.2). The co-slices were incubated for 3 weeks and the medium was changed once a week.

2.2 Preparation of collagen hydrogels

Collagen hydrogels were prepared as described previously [17]. Briefly, the collagen hydrogel was prepared by mixing 2 mg/ml bovine collagen type I (Collagen Solutions) with the crosslinker 0.4 mM poly(ethylene glycol) succinimidyl succinate (4S-StarPEG) (Sigma, JKA7006-1G) in phosphate-buffered saline (PBS). In order to generate GDNF loaded hydrogels, recombinant murine GDNF (Peprotech, 450-44-10) was added to the collagen hydrogel solution to the final concentration of 5 ng/ μL GDNF (pH 7.2). Empty collagen hydrogels were generated by adding the same volume of PBS to the hydrogel solution (pH 7.2). Droplets of 2 μL from the solution (containing 0 or 10 ng GDNF) were pipetted onto sterilized teflon tapes and incubated at 37°C for 1 h for gelation to occur.

2.3 Loading and release of GDNF into and from microspheres

Hollow microspheres were prepared via the Layer-by-Layer (LbL) approach using CaCO_3 microparticles as a sacrificial template and polyallylamine hydrochloride (PAH),

dextran sulfate (DEX) and tannic acid (TA) as polyelectrolytes, as described previously [21]. In this work, lyophilized microspheres were resuspended with a final concentration of 2.5 μg spheres/ μL in 0.1% Triton-PBS (T-PBS) with different concentrations of GDNF (0, 0.5, 1 and 5 $\mu\text{g}/\text{mL}$). These solutions were then incubated on a shaker at room temperature (RT) for 24 h to allow GDNF diffusion into the hollow microspheres. At the end of the incubation time, the solutions were centrifuged at 14000xg for 20 min in order to obtain the loaded microspheres. Resulting microsphere pellets was dissolved in PBS with 2.5 μg spheres/ μL concentration. This solution was either placed directly onto the gap between the co-slices (2 μL) or within collagen hydrogels (0.5 μg spheres/hydrogel) (Fig 3A). The release profile of the GDNF from the microspheres was evaluated as an acellular release system by incubating loaded microspheres (0.25 mg) in 100 μL slice medium at 37°C. The solutions were centrifuged at 14000xg for 20 min and supernatants were collected at days 3, 7, 14 and 21. Collected supernatants were stored at -20°C until further analysis. The cumulative release of GDNF was analysed by ELISA (FineTest, EM0088) according to the manufacturer's protocol.

2.4 Application of cryogels

Cryogels were prepared as described previously [25] but with a 1:1 molar ratio of PEGDA to the sulfonated monomer 3-sulfopropyl acrylate (SPA). These were produced in a PDMS template yielding line-shaped cryogels 10 mm long, 450 μm wide and 250 μm deep. These PEGDA-co-SPA cryogels were incubated at RT in PBS (empty cryogel controls) or 5 $\mu\text{g}/\text{mL}$ GDNF in PBS overnight for loading. Following incubation, the cryogels were taken with sterile tweezers and placed onto the co-culture across the slices with a gap as shown in Fig. 4A.

2.5 Microcontact printing

Silicon wafer master mold design and fabrication: The pattern for microcontact printing was designed and processed in Tanner L-Edit Software. It consisted of an array of 900 μm long lines with width of 30 μm running parallel to each other with 15 μm spaces in between. Silicon wafers with a layer of silicon oxide were used as a substrate for master mold fabrication. Micropatterns (4.7 μm high) were created using SU-8 photolithography. First, a layer of SU-8 2005 was spin coated at 5000 rpm for 1 min to obtain a layer of desired thickness. The silicon wafers then underwent a soft bake for 3 min at 90°C on a hot plate. Then, maskless aligner (MLA100, Heidelberg Instruments) was used to expose the design micropattern. The wafer was baked again at 90 °C for 3 min, developed in a bath containing the developer mr-Dev 600 for 10 min, and thoroughly rinsed with isopropanol.

Microcontact printing was performed as described before [27,30] but with modifications. Stamps were fabricated by casting and curing elastomer PDMS (Sylgard 184, Dowsil 184

Silicone elastomer kit, 01673921) against a micropatterned silicone wafer. An ink of antibody solution was prepared with anti-rabbit Alexa546 (Invitrogen) or antibody against GDNF (Abcam, Ab18956) with a concentration of 40 $\mu\text{g}/\text{mL}$. Approximately 100 μL of antibody solution was applied carefully onto each PDMS stamp, covering the entire printing surface. The stamps were incubated with antibody solutions for 20 min at RT in the dark and then excess solution was removed by filter paper without touching the printing surface and then washed twice with distilled water. After the second wash, the stamps were completely dried under a stream of nitrogen. The stamps were pressed against membranes placed on glass slides for 10 min with 5 g weight on top and incubated for 15 min. The position of the stamp was marked for later arrangement of the slices. The weight and the stamp were removed carefully. Membranes were sterilized under UV for 20 min. Membranes printed with anti-GDNF antibody were incubated with a GDNF solution with a concentration of 100 ng/mL in PBS overnight at 4°C on a shaker for loading of GDNF. Control membranes were incubated in PBS only. Membranes were washed 3x with PBS, equilibrated with slice medium and placed on inserts. Co-slices with a gap were prepared so that the printed pattern was between vMES and doSt (Fig. 7A).

2.6 Immunohistochemistry

Immunohistochemistry was performed as described previously [31]. Shortly, brain slices were fixed with 4% paraformaldehyde (PFA) solution for 3 h at 4°C. Slices were washed 3x with PBS and incubated for 30 min in T-PBS at RT. Slices were washed again and blocked with 20% horse serum/0.2% bovine serum albumin (BSA)/T-PBS for 30 min. Samples were incubated with primary antibodies against tyrosine hydroxylase (TH) (1:500, Proteintech, 25859-1-AP) or glial fibrillary acidic protein (GFAP) (1:2000, Millipore, AB5541) T-PBS/0.2% BSA at 4°C for 48 h. Following incubation, slices were washed 3x with PBS and incubated with Alexa488 conjugated anti-rabbit secondary antibody for TH and Alexa488 conjugated anti-chicken secondary antibody for GFAP. All slices were counterstained with DAPI (1:10,000). After a last washing step of 3x with PBS, the slices were mounted with Mowiol onto glass slides. Staining was visualized with a Leica DMIRB inverse microscope and Openlab software (4.0.4). Confocal microscopy was performed using an SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) with an HCX PL APO _63 and/or 1.3 NA glycerol objective and reconstructed with Imaris 8.2 software, as reported previously [32].

2.7. Western blotting

Western blotting was carried out as described previously [17]. Recombinant human and mouse GDNF (500 ng) were loaded onto 10% Bis-Tris polyacrylamide gel (Invitrogen) with 5 μL sample buffer. Electrophoresis was performed for 35 min at 200 V. Samples were electrotransferred onto PVDF membrane for 20 min at 25 V in semi-dry transfer cell (Thermo

Scientific). WesternBreeze Chemiluminescent immunodetection system (Invitrogen) was used for blotting. Blots were blocked with blocking buffer for 30 min and incubated overnight on a shaker at 4°C with primary antibody against GDNF (1:500). Blots were briefly washed and incubated with alkaline phosphatase-conjugated anti-rabbit antibody for 30 min at RT. Subsequently, blots were briefly washed again, incubated for 15 min in CDP-Star chemiluminescent substrate solution (Roche) and visualized with a cooled CCD camera (SearchLight, Thermo Scientific).

2.8. Data analysis and statistics

Slices were included in the study only if they were in the correct region, including a characteristic structure of vMES. Slices with <40 TH+ neurons were not included in the study, based on our previous study showing that the lowest number of TH+ neurons was 59 ± 19 ($n=8$) from the first sections of vMES after 2 weeks in culture [17]. The density of TH+ fibers was evaluated blinded similar to previous reports [33,34]. Briefly, 2-3 images at a 20x magnification were captured for each evaluated region. These images were taken from the border of vMES with doSt or Ctx for the co-slices. In the experiments with a gap between the slices the images were taken from the gap neighboring the vMES slice. In cryogel experiments 2 images were taken from both sides of the cryogel. The images were captured with the same mode and light settings for all evaluated slices. Images were analyzed in ImageJ by applying Otsu threshold and converting them into binary images (See Suppl. Fig.1). A value for percentage of pixels stained with TH antibody was calculated for each image, representing the area occupied by fibers in the focal plane. Image structures larger than a threshold value (40 pixels) were removed to eliminate non-specifically coloured cells with high background. The mean of the values from the 2-3 images for each brain slice was used for statistical analysis of the TH fiber intensity. All values are given as mean \pm standard error of the mean (SEM). The sample size (n) always specifies the number of slices evaluated that were taken from different animals. Statistical analysis was performed by one-way ANOVA with a subsequent Fisher LSD post-hoc test, where P -values lower than 0.05 represented significance.

3. Results

3.1. Co-cultures of vMES and doSt/Ctx

Co-cultures composed of ventral mesencephalon (vMES) and dorsal striatum (doSt) with cortex (Ctx) were made by obtaining coronal vibrosections from the respective areas, cutting these slices in half and placing them together (or with a small gap between) on the membranes (Fig. 1A). Brain slices were always counterstained with DAPI to discriminate tissue borders and brain regions (Fig. 1B). Slices were stained for tyrosine hydroxylase (TH) to visualize dopaminergic neurons of vMES and their fibers (Fig. 1C). Only healthy neurons with visible nuclei were counted (Fig. 1D). Dopaminergic neurons survived well in culture after 3 weeks of incubation.

3.2. Effects of collagen hydrogels

Co-cultures were incubated either with 100 ng/mL recombinant murine GDNF in the medium or with a collagen hydrogel applied at the tissue border of two half slices at the intersection of three brain regions. Collagen hydrogels were loaded either with PBS (control) or with 10 ng GDNF /hydrogel. The distance between the dopaminergic neurons at the vMES to the tissue border with the doSt/Ctx was 0.5 ± 0.2 mm (n=45). When co-cultures were incubated with GDNF in the medium, there was a general increase in the fiber density, including towards the border area (Fig. 2A). The placement of a control collagen hydrogel did not cause a notable increase in the density of fibers going towards doSt/Ctx (Fig. 2B). However, fiber growth towards the doSt/Ctx was pronounced for collagen hydrogels loaded with GDNF (Fig. 2C). The number of TH+ dopaminergic neurons was higher in co-cultures incubated with GDNF in the medium compared to single vMES slices incubated with 100 ng/mL GDNF in the medium or co-cultures with collagen hydrogels; however, these differences were not statistically significant (Fig. 2D). The density of the TH+ fibers increased at the border region towards both the doSt and Ctx when the slices were treated with GDNF compared to control co-cultures (incubated without a hydrogel and without GDNF in the medium). When a control (empty) hydrogel was placed in the border of co-cultures, the fiber density towards the doSt slightly increased compared to the control, while there was no difference in the fiber density towards the Ctx. The placement of a collagen hydrogel loaded with GDNF resulted in a significant increase in fiber density towards both the doSt and Ctx compared to an empty hydrogel (Fig. 2E).

3.3. Effects of microspheres

Co-cultures of vMES and doSt/Ctx were placed 0.7 ± 0.2 mm apart (n=15) onto the membranes. Microspheres loaded with GDNF were either pipetted into the gap between the

two half slices or incorporated into collagen hydrogels (Fig. 3A). The release of GDNF from the microspheres increased depending on the concentration of GDNF in the loading solution (0.5-5 $\mu\text{g/mL}$) (Fig. 3B). Co-cultures with empty or loaded microspheres in between the half slices did not survive well; some did not show thinning, indicating overall low survival, and some co-slices had less than 40 TH+ dopaminergic neurons, which were not included in the analyses. Co-cultures with a GDNF-loaded hydrogel in between exhibited TH+ fiber growth across the gap (Fig. 3C). For co-cultures with collagen hydrogels in the gap, the TH+ fiber densities were compared for the region that was within the hydrogel and for the region that was outside of the hydrogel. There were only a few fibers observed for co-cultures with an empty hydrogel in between. When a GDNF loaded hydrogel was placed between slices, the density of TH+ fibers outside of the hydrogel did not change while the density of the fibers within the hydrogel increased compared to the empty hydrogel group (Fig. 3D). Hydrogels with GDNF loaded microspheres exhibited a similar TH+ fiber density to the empty hydrogels (Fig. 3D).

3.4. Effects of cryogels

Cryogels were laid across the gap of the co-slices in a way that each end of the cryogel was on each half slice (Fig. 4A). Cryogels, which had a width of 450 μm , were easily visualized with bright field microscopy (Fig. 4B) and stained strongly with DAPI due to electrostatic loading. Cryogels, either empty or GDNF-loaded, induced significantly greater TH+ fiber growth from vMES than the control group of co-slices with nothing in between (Fig. 4C). However, loading of GDNF to cryogel did not cause a significant increase compared to empty cryogels (Fig. 4C). Most of the TH+ fibers appeared to adhere to the cryogel rather than growing towards the target region doSt. To investigate the host response to the material, slices were stained for reactive astrogliosis. Reactive GFAP+ astrogliosis slightly increased with time around the cryogels (Fig. 4D-F). Cryogels in combination with dopaminergic cells could be visualized by staining for DAPI together with TH (Fig. 4G). TH+ fibers adhered to the cryogel or grew along and parallel to the cryogels from vMES to the direction of doSt in co-cultures (Fig. 4H). In co-cultures combined with cryogels, fiber growth was difficult to study by inverted microscopy, as the TH fibers typically ran along the surface of the cryogels. No fibers were observed growing within the cryogels. 3D reconstruction of the cross-sections from confocal images of co-slices with cryogels revealed TH+ fibers attaching to the surface of cryogels (Fig. 5A-C). Several TH+ dot-like stainings were observed (Fig. 5D). Although some TH+ fibers grew along cryogels, they did not penetrate through the pores of the material but were attached to it and grew along the surface (Fig. 5E).

3.5. Effects of microcontact printing

The microcontact printing method was not successful for immobilization of recombinant GDNF directly onto the membrane (data not shown). Therefore, we employed an alternative approach in which an anti-GDNF antibody was printed onto the membrane and loaded with GDNF by incubating the membrane in 100 ng/mL GDNF solution overnight (Fig. 6A). To ascertain whether the printing procedure was successful, anti-mouse Alexa546 antibodies were printed onto membranes as controls (Fig. 6B). Microcontact printing of anti-GDNF antibodies alone as negative controls did not result in any fluorescence (Fig. 6C), while the printing pattern was clearly observed in the membranes printed with anti-GDNF antibodies (rabbit origin) and incubated with secondary anti-rabbit Alexa546 antibodies (Fig. 6D). To test the selectivity of the anti-GDNF antibody, Western blots were performed and showed a strong single band of an approx. 25 kDa for mouse and human recombinant GDNF (Fig. 6E).

Membranes were prepared by microcontact printing the respective antibody and arranging the co-cultures so that the printed pattern was in between half slices of vMES and doSt/Ctx (Fig. 7A). After 3 weeks in culture, dopaminergic neurons were evaluated by TH staining, during which the printed pattern was preserved. Control groups with printed anti-rabbit Alexa546 and anti-GDNF antibody alone resulted in almost no fiber growth between the slices after 3 weeks in culture. Printed anti-GDNF antibodies coupled with GDNF provided significantly more TH+ fiber growth between the slices compared to both control groups (Fig. 7B). As antibodies against both TH and GDNF were of rabbit origin, the printed anti-GDNF lines could be faintly observed via the fluorescent microscope. Fibers parallel to the printed lines could be observed in the anti-GDNF+GDNF group (Fig. 7C-F).

4. Discussion

In the present study we demonstrated the potential of different biomaterials, such as collagen hydrogels, microspheres, cryogels and microcontact printed surfaces, loaded with GDNF to repair the nigrostriatal pathway in *ex vivo* organotypic co-slice models.

4.1 Organotypic brain co-slices

Organotypic brain slices have been well established in our laboratory for nearly 20 years, including co-slices of coronal vibrosections of SN and doSt made with a tissue chopper [29] and also sagittal slices [35]. Although a sagittal slice model has the anatomical integrity and structures, this model in our experience does not contain an intact nigrostriatal pathway, as the cutting of the brain slices never follows the correct angle to allow intact nerve fibers to be present [35]. There are other studies showing sagittal models with slices of 300-400 μm thickness [7,36]. However, neuronal survival is dependent on initial tissue thickness. Slices with a large thickness do not flatten during culturing, which implies that the cells have low survival due to reduced diffusion of media and oxygen, besides inadequate waste removal [37]. Therefore, we chose a co-culture of thin (150 μm) coronal slices from the respective regions, proven to show good viability. In fact, present study represents also an extension of our previous work [17,33], by using the combination of two coronal brain slices to study not only the survival of dopaminergic neurons but also the nerve fiber innervation towards its natural target.

Dopaminergic neurons survived well after 3 weeks in culture, which is in agreement with other studies [11,12,17,38]. Healthy dopaminergic neurons in the brain slice cultures were distinguished with neuronal extensions and a clear cytoplasmic TH staining. The cut-off value of 40 TH+ neurons was decided based on our previous work, where the first slices in the target region included 59 ± 19 ($n=8$) dopaminergic neurons after 2 weeks of culturing [17] in order to use maximum numbers of slices in the correct region with reproducibility and precision. The evaluation of TH fibers in organotypic brain slices represents a challenge, as they do not appear as continuous solid structures, but instead present many varicosities [33,35]. Especially when they grow together as a bundle, it is not easy to discriminate individual fibers from each other. Some previous studies used a scoring system to evaluate fiber density [10,39] while others published camera lucida drawings to depict the TH+ fibers in whole [10,38]. We evaluated the fiber growth as the percentage of pixels stained by TH antibodies at the tissue borders, similar to a previous report [33]. Using this technique, we could clearly show TH+ nerve fiber growth to compare the effects of biomaterials added.

Unfortunately, the background staining in the target area (doSt/Ctx) was partly very high, so that we were not able to quantify nerve fiber growth within the target area. This was due to existing TH+ dopaminergic nerve terminals in doSt, which did not completely vanish after culturing. Additionally, at the end of 3 weeks culturing period Ctx part of the slices was generally thicker than the doSt or vMES, which caused strong background staining with immunohistochemistry. Consequently, we took advantage of a model with a small gap between the slices to better observe the presence and density of TH fibers. Taken together, the brain slices model of the vMES and doSt/Ctx is a useful tool to study effects of biomaterials on the growth of the nigrostriatal pathway with potential to study repair mechanisms.

4.2 Effects of collagen hydrogels on nerve fiber growth

Collagen and collagen hydrogels have been widely used to repair various tissues and structures including brain (see review [40]). Collagen hydrogels loaded with growth factors such as NGF and GDNF have been recently tested by us on organotypic brain slices [17,18]. In these studies, we showed that GDNF can be loaded into collagen hydrogels and be released in a time-dependent pattern, and we showed that GDNF-loaded collagen hydrogels provided neuroprotection to dopaminergic neurons in organotypic brain slices [17]. We further showed that these collagen hydrogels were not toxic when applied onto brain slices and exhibited only a minor reactive gliosis [17,18]. Our present data clearly show that the addition of a hydrogel did not influence the number of dopamine neurons after 3 weeks in culture. Herein, we wanted to extend our analysis to show that GDNF-loaded collagen hydrogels can also target nerve fiber growth of dopaminergic neurons. Our present data show that some spontaneous dopamine nerve fiber growth occurred towards their target area (doSt) even in the absence of any external stimulation such as addition of GDNF. This is in line with previous results [12,34], that the target area alone can stimulate a re-growth of dopamine nerve fibers. Addition of GDNF caused an increase in the fiber growth towards both doSt and Ctx, but this was a result of an overall increase in the fiber density all through the slice. Our data also provide evidence that the addition of a GDNF-loaded collagen hydrogel significantly increased the nerve fiber growth to both doSt and Ctx, which was even more predominant compared to GDNF in the medium. We observed a similar paradigm in our neuroprotection study with the same GDNF loaded collagen hydrogel system, in which lower amounts of GDNF loaded in the hydrogels (10 ng/ μ L) provided comparable effects with bolus GDNF application (100 ng/ μ L) via the culture medium due to slow and targeted release of loaded GDNF [34]. In this study, collagen hydrogels located on the slice provided a more

targeted fiber growth compared to GDNF in the medium, indicating the importance of localized drug delivery.

A similar effect was also seen when the GDNF-loaded hydrogel was placed in a gap in between the two brain slices. Overall, the fiber growth was higher when the slices were placed together compared to slices with a gap in between, which is due to anatomical support provided by the continuous tissue. However, hydrogels can create a favorable microenvironment with their mechanical similarity to soft tissues and have become attractive tools for neural tissue applications [41]. The positive effect of 3D collagen type I gels on neurite growth from various neuron types is well-documented [42,43]. Therefore, collagen hydrogels alone may provide an advantage for fiber growth as they provide an extracellular matrix-like structure and act as a supporting scaffold [44,45]. In this study, we found that the area in the gap that was within the empty collagen hydrogel contained slightly more nerve fibers than the area outside the hydrogel (Fig. 3D). In contrast, placing a collagen hydrogel loaded with GDNF in the gap between slices induced more extensive fiber growth within the hydrogel compared to control hydrogels, while fiber growth at the area outside hydrogel remained the same. These results demonstrate the merit of GDNF in this model and highlights the necessity of applying collagen hydrogel in combination with growth factors. Our data therefore clearly show that the application of a GDNF-loaded collagen hydrogel can be a potential tool to induce nerve fiber growth and to repair of the nigrostriatal pathway.

4.3 Effects of microspheres on nerve fiber growth

In the present study we used PAH-DEX-TA microspheres as described before in our group [21] and loaded these with GDNF. Indeed, we show that GDNF could be loaded and released from microspheres in a dose (0.5-5 μg) and time (0-21 days) dependent pattern, and we observed up to 14 ng/mL released GDNF into the medium. However, when we applied the microspheres directly into the gap between the two brain slices, the microspheres immediately diffused away into the medium or onto the brain slices. In these cases, also the dopaminergic neurons underwent cell death and not more than 40 neurons/slice were visible. In order to capture the microspheres and to prevent the cell death, we loaded them into the collagen hydrogel and placed this hydrogel in the gap between the two slices. Unfortunately, the GDNF-microsphere/collagen hydrogel method failed to provide any improvement in TH+ fiber growth compared to the hydrogels only. These results may be due to an insufficient amount of GDNF released from the composite material or a possible cytotoxicity of the materials chosen to the organotypic brain slice culture system. More work will be necessary to characterize the use of microspheres for local targeted delivery of GDNF in brain slices. For future studies, more compatible biomaterials can be used for the fabrication of

microspheres, combined with optimization of their GDNF loading/release profiles with and without a hydrogel.

4.4 Effects of cryogels on nerve fiber growth

The line-shaped cryogels used herein were 10 mm in length, 450 μm wide and 250 μm deep. This form of cryogel was easy to handle and could be easily placed between the two brain slices with the desired orientation. **A great advantage of cryogels compared to hydrogels is that they can be prepared in large quantities and stored for later use.** Furthermore, the cryogels could be easily visualized under the phase-contrast microscope. The intense cryogel fluorescence observed when incubated with DAPI can be attributed to the charge-charge interaction between the negatively charged sulfonate group of the cryogel and the multiple amine groups which become protonated in solution [46]. Further, when the cryogels were placed on brain tissue the reactive astrogliosis was weak and only present at the borders of the cryogel, seen as an increase of GFAP and only after two weeks. The present study is the first one to apply a trophic factor (GDNF) on brain slices using cryogels. We were not able to show any release of GDNF into the medium, but rather suggest that GDNF sticks on the outer surface of the cryogels.

In the present study we show that TH⁺ nerve fiber growth was dense in the proximity of the cryogel, but most fibers were attached to the cryogel itself. The TH⁺ fiber density in the gap in proximity to the cryogel increased significantly compared to control group (no cryogel) with spontaneous fiber growth. In no cases we saw that TH fibers entered the pores of the cryogel, but instead extended along the material, as these pores were probably too small for TH fiber in-growth. In order to get a better resolution of the nerve fiber growth on the cryogels, we used confocal microscopy. Indeed, we provide evidence that the TH⁺ nerve fibers can grow along the cryogel. The significant increase of fiber densities with both control and GDNF loaded cryogels shows a positive effect of the material alone. Cryogels may provide a large solid surface for the fibers to grow on. There are several *in vitro* and *ex vivo* assays available in order to evaluate nerve fiber growth, which are based on neuron growth/migration or fiber extension on surfaces especially when coated with adhesive molecules [47,48]. This may be the reason for significant differences in fiber growth between empty cryogels and controls. On the other hand, GDNF incorporation to cryogel provided a slight increase compared to the control cryogels, indicating that the additional factors are beneficial for nerve fiber growth using this approach. Thus, our data show that cryogels could be useful to connect brain regions, but more work needs to be done with cryogels with bigger pores in order to allow nerve fiber growth through and along the macroporous cryogels.

4.5 Effects of microcontact printing on nerve fiber growth

Microcontact printing is a soft lithography technique that has been used to immobilize proteins onto surfaces. Commonly used surfaces for microcontact printing of proteins include glass slides, gold, silicon, and silicon oxide [27]. Currently, there is only a single study that used polycarbonate membranes printed with extracellular matrix proteins to test cell adhesion, but by using heavy ion etched membranes [49]. Usually microcontact printing seems to be straightforward for many proteins, especially with clear even surfaces and using plasma activation, but it has not been demonstrated yet for membrane inserts with pores. Unfortunately, we were unable to microcontact print recombinant GDNF directly to the membranes. There may be various reasons for this, such as insufficient activation of the substrate (membrane) or the stamp for GDNF adsorption without plasma activation [50], disruption of GDNF molecule structure during printing procedure, or blockage of the anti-GDNF antibody recognition region. However, we were able to print antibodies to the membrane surface, and we show a positive print of anti-rabbit or anti-mouse antibodies fluorescently labelled with Alexa 546 on the membrane.

Consequently, we used a modified indirect version of the classical microcontact printing technique by printing the anti-GDNF antibody to the membrane and then loading this print with recombinant GDNF. This is a similar approach as reported by others, such as printing avidin and adding the protein of interest with a biotin group [27]. In another study, ephrin protein pre-clustered with its antibody was used for microcontact printing for axon guidance [51]. Indirect patterning of protein in such a way is more likely to preserve the native conformation of the printed protein, resulting in better protection of the biological function compared to direct microcontact printing [52].

In order to test nerve fiber growth of TH+ fibers towards the target area, we microcontact printed GDNF as a bridge directly between the 2 slices. Our analysis clearly shows that TH+ fibers grew along with the printed anti-GDNF/GDNF pattern, while no growth was observed in the antibody alone controls. Our immunostainings demonstrate that TH+ nerve axons preferentially grew along the printed GDNF regions. To our knowledge, our study is the first one to use microcontact printing with organotypic brain slice cultures, the first one to print GDNF on membranes and the first one to show targeted dopaminergic nerve fiber growth along such printed bars. The microcontact printing method had the advantage of providing stability of printed lines over time and very high spatial resolution. A main disadvantage of microcontact printing is the requirement of large amounts of anti-GDNF antibody and GDNF for the printing and loading processes, however both the ink and the loading solution with GDNF can be used multiple times, although this may affect the printing/loading efficiency. This technique has the potential to have more success with

application of multiple guidance cues together or generation of increasing GDNF gradients from donor to target region. Overall, our data provide a novel technique to combine organotypic brain slices with printed proteins on the membrane inserts, which can be used to study nerve axon guidance in brain slices.

4.6 Suggested mechanism of GDNF-related nerve fiber growth

GDNF acts by binding to its co-receptor GDNF family receptor $\alpha 1$ (GFR $\alpha 1$), followed by their binding with receptor tyrosine kinase Ret [53]. GDNF is a target-derived neurotrophic factor, which is not synthesized in vMES while both GFR $\alpha 1$ and Ret are produced in vMES [54]. In addition to that, GDNF interacts with the neural cell adhesion molecule (NCAM) to promote neurite outgrowth [55,56]. In our model, downstream pathways of Ret and NCAM that stimulate nerve fiber growth might be triggered by exogenous GDNF. We suggest that GDNF loaded into the biomaterials is slowly released and acts as a chemoattractant for dopaminergic neuron fibers with intact physiological receptors. Although GDNF is the most potent neurotrophic factor for dopaminergic neurons, inherent innervation of striatum involves a complex interaction of several chemoattractant and repulsive cues for precise orientation of the nigrostriatal pathway [57].

4.7 Translation to *in vivo*

Biomaterials used in this study may have various applications such as therapeutic interventions, tissue engineering and drug screening. In the context of therapeutic options, each approach presents its own advantages and disadvantages that would affect the feasibility of their applications. Injectable hydrogel systems have drawn attention as flexible drug/cell delivery platforms due to reduced invasiveness, easy and reproducible production and adjustable mechanical properties [58,59]. Microspheres and nanospheres have already been used commonly in numerous studies for drug, cell or gene delivery to the brain. Unfortunately, the microsphere application was not successful in the organotypic brain slice system we used due to excessive diffusion. Its *in vivo* application may limit a quick dispersion. In fact, GDNF loaded Poly(DL-lactide-co-glycolic acid) (PLGA) microspheres were successful to provide functional recovery and dopaminergic reinnervation of striatum in several studies [20,60,61]. Cryogels produced with shape-memory can also be applied by conventional syringes and it has gained attention for potential clinical applications. Initially cryogels may be more suitable for peripheral nerve injury due to easier application [62]. Nonetheless, clinical application of all these materials comes with safety concerns, besides issues with their infusion rate, ideal therapeutic dose and homogeneous distribution of GDNF

within the target tissue and invasiveness of stereotaxic surgery. Lastly, microcontact printing can be used in implantable soft electronics in the future in clinical context [63]. Currently it is a robust method for tissue engineering with its high spatial resolution that can allow manipulation of small cell populations very precisely and is useful as a brain-on-a-chip technology. However, the fundamental aim of this current study is to show proof-of-principle and to explore the potential use of these approaches in *ex vivo* drug testing and tissue engineering applications.

4.8 Limitations and future directions

In the present study we show proof-of-principle that different biomaterials are useful to repair the nigrostriatal pathway and provide diverse effects on TH+ nerve fiber growth in terms of fiber length and density. It has been understood that a neuroprotective or neuroregenerative approach with GDNF should encompass a bigger picture of the whole nigrostriatal system rather than focusing solely on survival of dopaminergic neurons. The application of these techniques in clinical setting is also challenging due to increased distances between target regions in the human brain, intrinsic mechanisms that inhibit axon growth, safety and biocompatibility issues in the body and long-distance diffusion of trophic factor GDNF [41, 42]. A beneficial approach can be application of a cocktail of different factors such as antioxidants, anti-inflammatory molecules, guidance cues (netrin1) and other growth factors (brain-derived neurotrophic factor), which may become a more effective strategy to re-establish the degenerating networks [43]. Additionally, approaches to re-establish the nigrostriatal pathway can be also used in combination with transplanted dopaminergic neurons, as existing axons along the pathway guide the developing axons towards their target area [5].

Organotypic brain slices provide a valuable tool to study mechanisms and possible therapeutic approaches with straightforward manipulation of the experimental conditions and well-established analysis methods. A limitation of this study was the use of young wild type animals for testing of the biomaterials. In fact, the innervation from vMES to the striatum starts as early as embryonic day 14 [57]. As mentioned previously the use of young postnatal mice is crucial for organotypic brain slices to guarantee the survival of neurons. A future direction of this study should be studying these materials with organotypic brain slices taken from adult mice with PD markers for a more accurate representation. However, our present organotypic brain slice model displays retrograde degeneration of dopaminergic neurons due severed axons from slicing procedure, which can as well recreate an important element of the PD pathology.

5. Conclusions

Taken together, our study shows that organotypic co-slices of vMES and doSt/Ctx are an excellent model to study dopaminergic neurons and fiber growth. Application of materials for targeted trophic factor delivery can present future possibilities for regenerative therapy strategies. In our present study, collagen hydrogels loaded with GDNF were potent to enhance dopaminergic fiber growth to targeted and non-targeted brain regions. The use of microspheres on organotypic co-slices was challenging but did not work under the configuration presented in this study. Cryogels loaded with GDNF stimulated attachment and growth of dopaminergic nerve fibers. And finally, we show for the first time the microcontact printing of GDNF onto membranes and growth of TH+ nerve fibers along those prints. The use of microcontact printing together with organotypic slices may help for the construction of new neuronal networks and could be of interest in defining organoids or minibrains. In conclusion, different biomaterials have the potential to repair the nigrostriatal pathway, especially in organotypic brain slices. This study presents a tool kit for further testing of various biomaterial approaches in different models for better understanding of dopaminergic innervation and repair strategies for the nigrostriatal pathway.

Data availability

All data analyzed during this study are included in this published article. Other raw data required to reproduce these findings are available from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare no competing interests.

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Figure Legends

Fig. 1. Overview. Brain vibrosections were prepared from postnatal day 9-11 mice and cultured for 3 weeks. Co-slices were made by placing a slice containing the dorsal striatum (doSt) and surrounding cortex (Ctx) together with a slice containing the ventral mesencephalon (vMES) on a 0.4 μm membrane insert (A). All brain slices were labelled with nuclear DAPI (blue) to distinguish tissue borders (B) and immunohistochemically stained with antibodies against tyrosine hydroxylase (TH) to label dopaminergic neurons (green) (C). Only healthy TH⁺ neurons (green) with a visible DAPI⁺ nucleus (blue) were counted for quantification (D). Scale bar in B: 300 μm (B&C), 30 μm (D).

Fig. 2. Effects of collagen hydrogels on nerve fiber growth. Co-slices of vMES and doSt/Ctx were incubated with glial cell-line derived neurotrophic factor (GDNF, 100 ng/mL) in media (A). Alternatively, collagen hydrogels loaded without (B) or with GDNF (C) were placed directly at the border between the two slices. TH⁺ nerve fibers (green) grew towards the GDNF-loaded hydrogel (C), while not much of fiber growth towards the targets were seen in slices loaded with an empty hydrogel (B) or when GDNF was added to the medium (A) after 3 weeks culturing. The border of the two slices is shown by a dotted white line and the location of the hydrogel by a dotted white circle. White arrows indicate main location of cell bodies. The number of TH⁺ dopaminergic neurons was counted (D) and the density of TH⁺ nerve fiber growth was assessed by computer-assisted imaging at the slice border (E). Minus indicates a negative control with no GDNF or hydrogel. Values are given as mean \pm SEM number of TH⁺ neurons (D) or pixels (E), values in parenthesis give the number of analyzed mice. Statistical analysis was performed by One Way ANOVA with a Fisher-LSD post hoc test (* $p < 0.05$; ** $p < 0.01$);). No indication of significance in the graph means not statistically significant. Scale bar in C: 300 μm (A,B,C).

Fig. 3. Effects of microspheres on nerve fiber growth. Co-slices of vMES and doSt/Ctx were prepared with a gap in between. A collagen hydrogel loaded with GDNF (A, left) or loaded with GDNF-microspheres (A, right) was placed into the gap. Alternatively, GDNF-microspheres were placed into the gap without a hydrogel (A, middle). The release of GDNF loaded into microspheres (0.5-1-5 μg ; $n=1$ per time point and concentration) was measured in the medium at days 0-3-7-14 and 21 by ELISA and is given in pg/mL (B). After 3 weeks in culture, TH⁺ fibers were observed in the location where GDNF loaded hydrogel was placed (green, C). The gap between the two slices is marked with a white dotted line (C). The TH⁺

nerve fiber density was evaluated by computer-assisted imaging at the regions inside or outside the initial location of hydrogels (D). Values are given as mean \pm SEM pixels, values in parenthesis give the number of analyzed mice. Statistical analysis was performed by Student's T test or One Way ANOVA with a Fisher-LSD post hoc test (N/A could not be analyzed). No indication of significance in the graph means not statistically significant, only a trend ($p=0.07$) was found. Scale bar in C: 280 μm (C).

Fig. 4. *Effects of cryogel on nerve fiber growth.* Co-slices of vMES and doSt/Ctx were prepared with a gap in between. A cryogel with 450 μm width loaded with or without GDNF was placed in between the slices and cultured for 3 weeks (A). The cryogel was clearly visualized under dark field microscopy (B). Placing a cryogel that was empty or loaded with GDNF provided more TH+ fiber growth compared to control slices as measured by computer-assisted imaging (C). Values are given as mean \pm SEM pixels, values in parenthesis give the number of analyzed mice. Statistical analysis was performed by One Way ANOVA with a Fisher-LSD post hoc test ($***p < 0.001$). Reactive astrogliosis was evaluated by staining with antibodies against glial-fibrillary acidic protein (GFAP) at day 4 (D), day 11 (E) and day 14 (F). Note a slight reactive astrogliosis around the cryogel after 2 weeks (F). The border of the cryogel (*) is marked with a dotted white line (D-F). Figure G gives a general overview of a cryogel placed between co-slices (counterstained with blue DAPI) and co-localized with green fluorescent TH+ nerve fibers. The gap between the two slices is indicated by a white dotted line (G). Figure H shows TH+ nerve fibers adhering to or growing along and parallel to the cryogel. Scale bar in G: 340 μm (B), 100 μm (D-F), 450 μm (G), 150 μm (H).

Fig. 5. *Confocal images of cryogels.* Cryogels (loaded with GDNF) placed between two brain slices. After 3 weeks in culture, slices were fixed and immunohistochemically stained for TH (green) and counterstained with DAPI (blue). Note that cryogels display autofluorescence when incubated with DAPI, which allows visualization (A). Dopaminergic TH+ nerve fibers grew onto the cryogel (B&C) or attached to the cryogel forming strong TH+ like dots (D). Figure E depicts a TH+ dopaminergic fiber growing along a cryogel. Scale bar in A: 15 μm (A-D) and 10 μm (E).

Fig. 6. *Characterization of the microcontact printing method.* Microcontact printing was carried out by printing an anti-GDNF antibody on the membrane, which was either visualised by a secondary α -rabbit Alexa546 antibody or loaded with recombinant GDNF (A).

Membranes printed with α -rabbit Alexa546 antibody (B) showed a red fluorescent staining pattern, while a control membrane printed with only anti-GDNF antibody shows only background (C). Membranes printed with α -GDNF antibody and then stained with secondary anti-rabbit Alexa546 antibody shows again a positive red printing pattern (D). Western Blot analysis confirms that the α -GDNF antibody recognized human and mouse GDNF as a single band of approx. 25 kDa (E). Scale bar in C: 150 μ m (B-D).

Fig. 7. *Effects of microcontact printing of GDNF on nerve fiber growth.* GDNF was microcontact printed (anti-GDNF+GDNF) on membrane inserts and co-slices of vMES and doSt/Ctx placed in between. As a control the antibodies alone (anti-GDNF or anti-rabbit-Alexa 546) were printed. After 3 weeks in culture, slices stained for TH and dopaminergic fiber density was evaluated by computer-assisted imaging (B). Values are given as mean \pm SEM pixels, values in parenthesis give the number of analyzed mice. Statistical analysis was performed by One Way ANOVA with a Fisher-LSD post hoc test ($*p < 0.05$). Note a clear and strong TH+ nerve fiber growth along the microcontact GDNF prints (C), while no fiber growth occurred in the control group. Figure E shows a higher magnification with a clear axonal TH+ nerve fiber growth following the printed GDNF line compared to a close-up control with no fiber growth (F) Scale bar in C: 250 μ m (C,D), 100 μ m (E,F). For better visualization Figures G and H show a close up (red arrows) and an artificial link to the microprints.