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Cocultures of Rat Sensorimotor Cortex and Spinal Cord Slices to Investigate Corticospinal Tract Sprouting

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Study Design. Experimental study of corticospinal axonal sprouting in an organotypic slice culture model.

Objective. To develop an *in vitro* model that simplifies the study of various factors regulating neuronal regeneration.

Summary of Background Data. Spinal cord injury leads to permanent neurologic damage, mainly due to the inability of the adult central nervous system to regenerate. Much attention has been focused on promoting axonal regeneration and sprouting, either by exogenous administration of various neurotrophic factors or by the antagonization of factors inhibiting regeneration.

Methods. An *in vitro* system that allows coculture of slices from rat sensorimotor cortex and spinal cord (p4) was established. Two groups of cultures were investigated: In the first group, intact spinal cord slices were cultured adjacent to sensorimotor cortex slices, while in the second group the spinal cord slices were sagittally cut into halves, with the sectioned interface placed directly adjacent to the sensorimotor cortex, to prevent the spinal white matter from interference. Each group was further divided into 2 subgroups: The neurotrophin-3 (NT-3) group, where the culture medium contained 50 ng/mL NT-3 and the control group treated with normal culture medium. Sensorimotor cortex pyramidal neurons were anterogradely labeled with Mini-Ruby, a 10 kD biotinylated dextran amine.

Results. Cocultures of cortical and spinal cord tissue were propagated *in vitro*, and axonal sprouting occurred. The group of cocultures treated with NT-3 showed an improved cortical cytoarchitecture, and sprouting axons were more frequently observed. In NT-3-treated cocultures where spinal cord gray matter was directly opposed to cortical slices sprouting axons entered the adjacent spinal cord tissue. This phenomenon was not observed if spinal cord pia mater and white matter were opposed to the cortical slices, or if NT-3 was absent.

Conclusion. Our data suggest that the absence of repellent factors such as white matter and the presence of neurotrophic factors promote axonal sprouting. Cocultures of sensorimotor cortex and spinal cord slices combined with anterograde axonal labeling could provide a valuable *in vitro* model for the simplified screening of factors influencing corticospinal tract regeneration.

Key words: sprouting, corticospinal tract, sensorimotor cortex, neurotrophin-3 spinal cord slice culture, Mini-Ruby. **Spine 2009;34:2494–2499**

Spinal cord injury is associated with irreversible damage to long fiber tracts such as the corticospinal tract, resulting in permanent para- or tetraplegia. The incurability of the condition is mainly due to the limited capacity of adult central nervous system neurons for axonal regrowth. This acquired inability to regenerate is mainly attributed to 2 factors: (i) the lack of adequate trophic support, and (*ii*), the presence of an environment that is hostile to regrowth.[1,2]

In an effort to overcome the limited regeneration in the lesioned central nervous system (CNS), much attention has been focused on promoting axonal regeneration and sprouting, either by exogenous administration of various neurotrophic factors or by the antagonization of factors that inhibit regeneration. Neurotrophic factors represent proteins that regulate neuronal survival, differentiation, axonal outgrowth, synaptic plasticity, and neurotransmission. Several groups of neurotrophic factors have been identified: Nerve growth factor, brain-derived neurotrophic factor, and neurotrophins (NT)−3,−4, and −5 are examples for factors that have been well characterized *in vivo* and *in vitro*. Experimental evidence suggests that neurotrophins such as NT-3 may be effective in the treatment of spinal cord injury in adult rodents.[3] Recently a rat organotypic coculture model was used for assessment of the role of age and neurotrophic factors.[4,5] Great insight into mechanisms that inhibit neuronal regeneration has been gained by experiments that defined repellent molecules such as Nogo.[6] Experimental removal of such obstacles has been attempted by the use of antibodies or enzymes directed against these molecules, sometimes with the result of enhanced neuronal regeneration or even improved function.[7]

The majority of experiments in the field have been carried out *in vivo*, which raises a number of experimental and ethical issues. We therefore set out to establish an *in vitro* model that simplifies the investigation of axonal sprouting under various experimental conditions. Organo-

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typic slice cultures of different CNS regions have become important tools to study degeneration and regeneration in the CNS, and we recently refined and characterized slice cultures from rat spinal cord where survival of defined neuronal and glial populations was found.[8] To study the potential of various neurotrophic or antirepellent factors to induce axonal sprouting we designed an *in vitro* model that comprises the origin and target regions of the corticospinal tract, that is, sensorimotor cortex and spinal cord. In this study, we therefore combined cocultures of rat sensorimotor cortex and spinal cord slices with anterograde axonal labeling. This coculture system was then applied to gain preliminary insight into the effects of pia mater and white matter removal, and of exogenously administered NT-3 on neuronal survival and axonal sprouting.

■ Materials and Methods

Preparation and Culture of Rat Cortex and Spinal Cord Cocultures

Wistar rats were raised and provided by the "Zentrale Forschungseinrichtung" of the Hospital of the Johann Wolfgang Goethe-University in Frankfurt am Main, Germany, according to the local guidelines for animal welfare. Tissue preparation was performed under aseptic conditions using sterile instruments. The following sterile instruments were used: 1 pair of large, bandage scissors, 2 pairs of small, straight, sharp, operating scissors, 2 fine surgical forceps, 1 fine, straight anatomic forceps, 2 curved anatomic forceps, 2 scalpel handles, 1 surgical blade no. 15, 1 surgical blade no. 11, 1 halved razor blade, 1 glass Pasteur pipette, 2 glass Pasteur pipettes with sawed off tip, and 2 round filters (diameter, 55 mm).

Cortical Slice Cultures. In order to produce Agar-blocks of required thickness and solidity for the slice culture preparation the following ingredients (all from Merck, Darmstadt, Germany) were diluted in 100 mL Aqua dest: 0.725 g NaCl, 0.0373 g KCl, 0.01725 g $NaH_2PO_4 \cdot H_2O$, 0.0493 g $MgSO_4$ $7H_2O$, 0.2184 g $NaHCO_3$, 0.18 g Glucose, 0.0294 g $CaCl_2$ $2H_2O$, 5 g Agar. The solution was heated for 30 minutes to 70°C on a magnetic stirrer and aliquoted into cell culture dishes (35-mm diameter), that after cooling were tightly sealed and stored at 4°C for subsequent sectioning. Before the preparation procedure, 2 Agar-blocks dimensioned to the length and width of the rat brain were fixed onto a vibratome (Vibratome 1000 Classic; TPI St. Louis, MO) Plexiglas insert with Histoacryl tissue-glue (Braun, Melsungen, Germany), forming an "L" configuration. A third agar-block was glued to the Plexiglas-insert after the brain explant had been positioned in the Plexiglas insert in order to achieve maximal stabilization during sectioning. The final Agar construct thus formed a "U" configuration that was open on the side facing the vibratome razor blade.

Coronal slice cultures from prefrontal cortex were subsequently obtained from p4 Wistar rats. After decapitation with large bandage scissors, the scalp was removed using small scissors and surgical forceps. With small scissors and surgical forceps, the calvaria was opened and removed, revealing the underlying brain. With a no. 11 scalpel the frontal pole and the cerebellum were removed and the brains were immediately placed in ice-cold preparation medium. After drying on a filter for a few seconds, the brain was carefully transported with the curved anatomic forceps onto the vibratome Plexiglas insert, where it was secured with Histoacryl tissue-glue and embedded between the agar blocks as described above. After attaching the halved razor blade to the sliding vibratome and filling the Plexiglas inset with 50 mL cold preparation medium, 4 to six 400-μm coronal sections were obtained from each brain. Preparation medium consisted of minimal essential medium (Gibco, Eggenstein, Germany), containing 1% glutamine (Gibco) with a pH = 7.35. Throughout the preparation process preparation medium was kept on ice, in order to maintain a temperature of approximately 4°C. Using the anatomic forceps and a no. 15 scalpel, the remnants of the surrounding dura mater were removed under microscopic control, and the brain slices were transferred into cell cultures inserts that were placed in 6 well culture dishes, containing 1 mL of culture medium per well. Culture medium consisted of 50% minimal essential medium, 25% Hank balanced salt solution (Gibco), 25% normal horse serum (Gibco), 2% glutamine, 1 μg/mL insulin (Boehringer, Mannheim, Germany), 2.4 mg/mL glucose (Braun), 0.1 mg/mL streptomycin (Sigma Chemicals, Deisenhofen, Germany), 100 U/mL penicillin (Sigma), and 0.8 μg/mL vitamin C (Sigma), with the pH adjusted to 7.4. The culture dishes were kept in an incubator during the following preparation of the spinal cord slices.

Spinal Cord Slice Cultures. Before preparation, Agar-blocks were prepared as follows: An Agar-block measuring approximately $2 \times 1.5 \times 1$ cm^3 was sectioned from the previously produced Agar aliquots and placed on a sterile round filter. Using the tip of a Pasteur pipette, 2 tunnels that were dimensioned to fit the diameter of the rat spinal cord were prepared. Subsequently, the Agar-block was bisected by using the scalpel blade no. 11, with the cut passing through the channels that had been opened before (Figures 1A, B). One half was placed horizontally, in order to accommodate the subsequently prepared spinal cord slices.

Transverse slice cultures of rat spinal cord were then obtained from p4 Wistar rats as follows: After decapitation with the large bandage scissors, the dorsal skin and musculature of the trunk were removed along the midline, using small scissors and surgical forceps. Subsequently, by use of the other set of small scissors and surgical forceps, a longitudinal laminectomy was performed from the cervical to the lumbar region of the vertebral column, the dura mater was incised, the spinal cord dissected from the denticulate ligaments and immediately placed in ice cold preparation medium (composition described earlier in the text).

Using the anatomic forceps and the no. 15 scalpel, the remnants of the surrounding dura mater were removed under microscopic control, and the spinal cord was cut into 2 segments: 1 cervicothoracic and 1 thoracolumbar. Both tissue segments were sandwiched between the 2 agar half-blocks described above, and the whole block was transferred onto the vibratome Plexiglas-insert, where it was secured with Histoacryl tissue-glue. This construct provided tissue protection and mechanical stabilization, reduced shear stress during sectioning and produced slices of uniform thickness. The halved razor blade was attached to the sliding vibratome, the Plexiglas-insert was filled with 50-mL ice cold preparation medium and the spinal cord was cut into transverse slices of 400-μm thickness. From each spinal cord 10 to 20 slices were obtained and transferred into cell cultures inserts (pore size, 0.4 μm) that were placed in 6-well culture dishes. Under microscopic control, transverse spinal cord slices were placed adjacent to the coronal brain sections, and the culture dishes were incubated at 35°C in a fully humidified atmosphere with 5% CO_2 in the culture medium described above.

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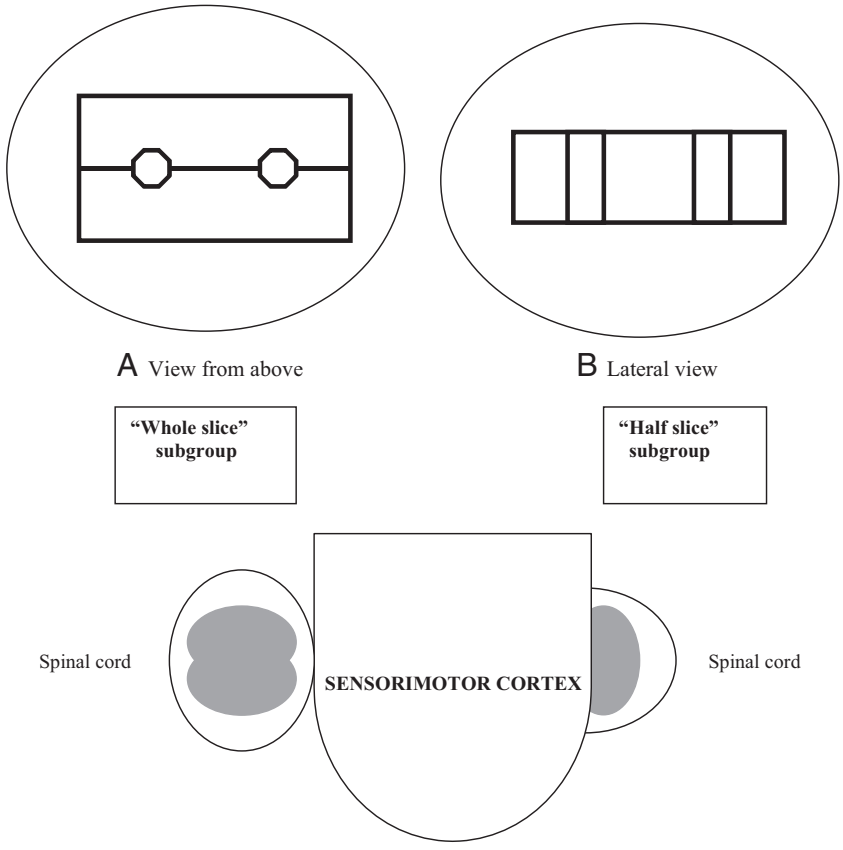

Figure 1. **A, B,** Schematic drawing of Agar-blocks preparation seen from above (**A**) and from the side (**B**). **C,** Schematic drawing of sensorimotor cortex and spinal cord slice cultures and their orientation in the whole slice and half slice subgroups.

Experimental Groups and Subgroups

The cocultures were divided into 2 groups. In the first group, intact spinal cord slices cultured adjacent to sensorimotor cortex slices were used ("whole slice" subgroup), while in the second subgroup the spinal cord slices were sagittally cut into halves under microscopic control, with the sectioned interface placed directly adjacent to the sensorimotor cortex ("half slice" subgroup; Figure 1C). This protocol was used in order to prevent the spinal white matter from interfering between the cortical and spinal gray matters. Each group was further divided into 2 subgroups: The NT-3 group, where the culture medium contained 50 ng/mL NT-3 (rhNT-3; R&D Systems, Wiesbaden, Germany), and the control group treated with normal culture medium as described above.

Anterograde Labeling of Sprouting Axons

One hour after preparation, neurons situated in the sensorimotor cortex were anterogradely labeled with Mini-Ruby (Molecular Probes, Eugene, OR), a 10 kD biotinylated dextran amine that is anterogradely transported in intact axons. Mini-Ruby was applied by placing a minute crystal of the undiluted tracer on the surface of the cortical slice culture, and Mini-Ruby fluorescence could be repetitively observed under an inverse light and fluorescence microscope (excitation filter, 520–550 nm). The culture dishes were immediately returned to the incubator, and the culture medium was changed every second day.

Fixation of Rat Cortex and Spinal Cord Cocultures and Immunohistochemistry

After 4 days *in vitro* (div) the cocultures were fixed with 4% paraformaldehyde (Merck), 15% picric acid (Merck) and 0.1% glutaraldehyde (Sigma) in 0.1 M phosphate buffer (PB) for 15 minutes, washed with 0.1 M PB and postfixed in the same fixative without glutaraldehyde for 1 hour. Cocultures were finally washed with 0.1 M PB for 1 hour, until the yellow color of the picric acid was completely washed away, and were carefully removed from the culture membrane using a fine paint brush. The slices were then placed in 0.8 M sucrose solution (Merck) containing 1.5% NaN_3 (Merck) and stored at 4°C.

After pretreatment with methanol and H_2O_2 (0.45%), the cocultures were washed with phosphate-buffered saline (PBS) and incubated with avidin-biotin-complex (Vector Laboratories, Burlingame, CA; diluted 1:100 in PBS with 0.3% Triton) for 1 hour. After thorough washing with PBS (2 × 10 minutes) and Tris-buffer (1 × 10 minutes), 3, 3′-diaminobenzidine (DAB; Sigma) was used as a chromogene (0.1 g DAB diluted in 200 mL Tris-buffer and activated with 100 μL H_2O_2 [40%]). The reaction proceeded under visual control with a light microscope, and when coloration became visible the reaction was stopped with Tris-buffer for 10 minutes. After washing, cocultures were mounted on gelatin-coated glass slides, counterstained with hematoxylin (AppliChem, Darmstadt, Germany), dehydrated through a graded series of ethanol and xylol and coverslipped with Entellan (Merck).

■ Results

In the groups of cocultures incubated in control medium and fixed after 4 div, the cytoarchitecture was poorly preserved in the sensorimotor cortex, while the spinal cord slice cultures were better preserved. In the spinal

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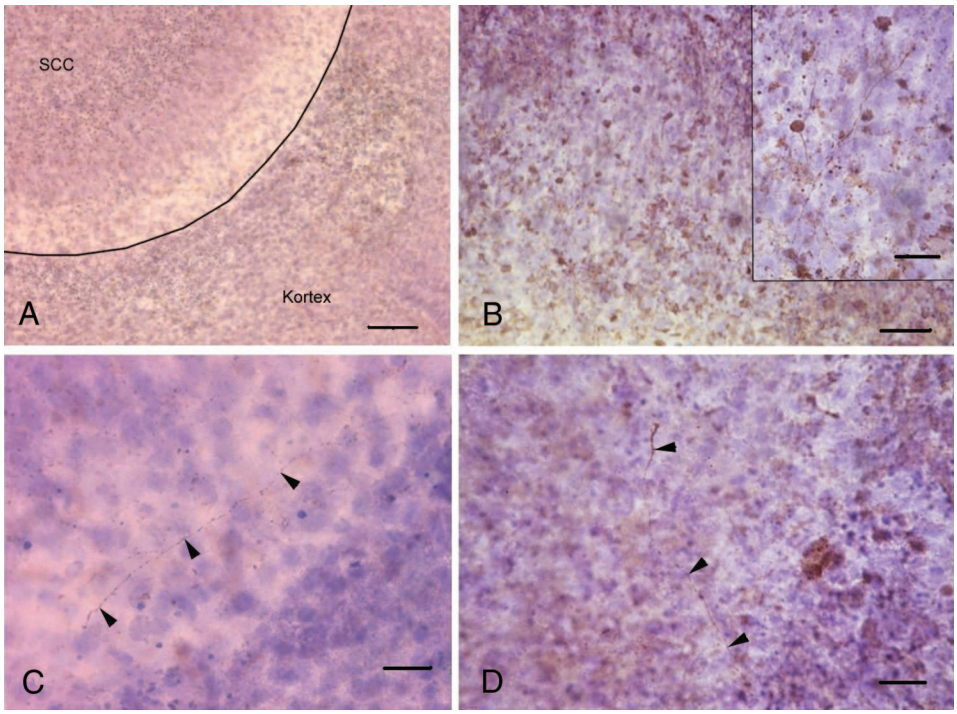

Figure 2. **A,** Slices from sensorimotor cortex were cocultured with spinal cord slice cultures. After 4 div, an interface connecting the cortical with the spinal cord slice had developed (line denoting the interface). **B,** In the group of cocultures treated with NT-3, numerous large neurons in the sensorimotor cortex that mostly resembled pyramidal neurons could be labeled by Mini-Ruby. In association with these labeled neurons, a network of dendrites and, in some instances, sprouting axons, could be observed. The insert shows a larger magnification of this sensorimotor cortex region, and a labeled axon with a growth cone that grows towards the interface region. The cortical surface is situated at the bottom, the interface region connecting the cortical culture to the spinal cord slice cultures would be beyond the upper border of the picture. **C,** In the group of whole slice cocultures treated with NT-3, axons (arrowheads) grew towards the corticospinal interface, finally demonstrating a course parallel to it, but not penetrating the white matter of the spinal cord culture itself. The cortical part of the coculture is situated at the bottom right side of the picture. **D,** In the group of half slice cocultures (treated with NT-3) where spinal cord gray matter was directly opposed to the cortical cultures, sprouting axons traversed the corticospinal interface and entered the gray matter of the spinal cord. Sprouting axons (arrowheads) with growth cones displayed a maximal length of 500 μm. The cortical part of the coculture is situated at the bottom right side of the picture. All preparations counterstained with hematoxylin. Scale bar in **A**: 200 μm, **B**: 400 μm (insert: 40 μm), **C**, **D**: 40 μm.

cord ventral horns, cells of a size corresponding with small and medium size motoneurons were visible, whereas large motoneurons seemed to be missing. Large quantities of dorsal horn interneurons were found to persist. After 4 div, an interface connecting the cortical and the spinal cord slice culture had developed, bridging the small gap between the 2 slice cultures that initially existed (Figure 2A). Axonal labeling following Mini-Ruby application on the sensorimotor cortex was visible in fluorescence microscopy during the period of *in vitro* culture, and after fixation and preparation for light microscopy after 4 div. We observed very few labeled axons, originating from the sensorimotor cortex, in the area surrounding the corticospinal interface of the group of whole slice cocultures. Sprouting axons were rare, short, and limited to the area of the sensorimotor cortex. No sprouting axons actually crossed the corticospinal interface or entered the spinal cord tissue. In the group of half-slice cocultures where spinal cord gray matter was directly opposed to the cortical cultures and culture was performed in control medium there was no obvious improvement in the amount of axonal sprouting.

In contrast, in cocultures treated with NT-3, an improved survival of cortical neurons was seen. Numerous Mini-Ruby-labeled neurons were found in all layers of the sensorimotor cortex. They were accompanied by a fiber network consisting of Mini-Ruby labeled dendrites and axons (Figure 2B). As in cocultures treated with control medium, the preservation of spinal cord morphology was generally good. Anterograde labeling of neurons in the sensorimotor cortex resulted in visualization of sprouting axons that were longer than in the control group. In the area of the corticospinal interface, sprouting Mini-Ruby-labeled axons were observed. In the group of whole slice cocultures, these axons grew towards the corticospinal interface, but changed their course and eventually ran parallel to the interface (Figure 2C). They were unable to cross the corticospinal interface, consisting of pia mater and white matter, in order to enter the spinal cord. In contrast, in the group of half slice cocultures treated with NT-3, some sprouting axons actually crossed the corticospinal interface and entered the spinal cord tissue. In this subgroup, sprouting axons with growth cones were seen (Figure 2D).

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■ Discussion

The riddle of insufficient recovery after SCI is currently studied by approaches that aim at identifying factors that promote or inhibit neuronal and axonal regeneration. Mostly, these studies are carried out *in vivo* in animal experiments. It was our aim to develop a relatively simple *in vitro* model that allows for the investigation of axonal regeneration in the corticospinal system, enabling screening of a multitude of factors that have the potential to promote regeneration. We therefore established a coculture model of sensorimotor cortex and spinal cord slices.

The ability of corticospinal neurons to survive axotomy appears to be well correlated with their innervation of spinal targets. In rodents, neurons die if they are axotomized before target innervation, but are able to survive if axotomy occurs after they have innervated spinal targets. Axotomy after an age of 14 days or more causes cell shrinkage but not cell death. Furthermore, it has been reported that axotomy of corticospinal axons in the adult rat close to the *perikarya*, that is, at the level of the internal capsule, resulted in the demise of many corticospinal neurons. Since our slice culture preparation equals a severe proximal axotomy and the cultures were derived from p4 animals, the observed pyramidal cell death in the control group was an expected finding.

The number and morphology of smaller $NeuN^+$ neurons in the gray matter of spinal cord slice cultures have been found to remain well preserved, even after prolonged culture periods.[8] $Calbindin^+$ neurons also showed a capacity for survival over prolonged culture periods, although their numbers decline with time. This relatively high degree of preservation of smaller $NeuN^+$ or $calbindin^+$ neurons in spinal cord slice cultures is contrasted by an early loss of large motoneurons in the ventral horn. The early loss of ventral horn motoneurons observed in slice cultures correlates well with the observation that immature motoneurons are vulnerable to target deprivation.[9]

The improved survival of cortical neurons in the NT-3-treated group indicates a potent neuroprotective effect of NT-3. There are numerous *in vivo* experiments demonstrating that NT-3 prevents axotomy-induced cell death of corticospinal neurons.[10] In the NT-3-treated group, labeled corticospinal sprouting axons were observed in the corticospinal interface, while this phenomenon was not observed in the control group. *In vivo*, NT-3 has been found to enhance regeneration and sprouting of corticospinal axons both during development and after adult spinal cord lesion, alone or combined with other neurotrophins.[1,3,11] In a similar organotypic coculture model neurotrophic factors secreted by transplanted neural progenitor cells promoted corticospinal axonal growth.[4] We observed that regenerating axons were unable to penetrate the spinal cord tissue in the whole slice subgroup, whereas the half slice subgroup contained sprouting axons that crossed the corticospinal interface and entered the spinal cord tissue. This observation is possibly explained by "Nogo"—molecules expressed on oligodendrocytes, and it is in accordance with *in vivo* findings: Regeneration and sprouting of axons after an experimental spinal cord lesion and subsequent tissue grafting occurred almost exclusively within the remaining gray matter at the lesion area while it avoided the white matter.[11–13] Thus, it appears that the presence of oligodendrocytes or white matter inhibits axonal regrowth even in the presence of neurotrophic factors, whereas neurotrophin-induced axonal sprouting can take place when white matter has been abolished. This hypothesis is strengthened by observations on enhanced axonal sprouting after elimination of white matter[14] or after combined application of NT-3 and an antibody that neutralizes neurite growth inhibitors.[15]

There are many limitations to the *in vitro* model described here: Primarily, the loss of neurons both in the cortex and in the spinal cord subsequent to culture preparation represents a severe degenerative alteration. However, such early neuronal loss is well known from other slice cultures such as organotypic hippocampal slice cultures. In such preparations, the loss of granule and pyramidal cells is marked during the early phase of the culture period,[16] but keeping this limitation in mind, that model has proven to be of great value.[17] A further limitation of our coculture system is the immaturity of the tissue that is used to produce the slice cultures: It was earlier described that the highest degree of neuronal and cytoarchitecture preservation in spinal cord slice cultures is achieved when animals of an age up to p3 are used as a source of tissue, whereas cultures derived from animals of age p6 and older are of inferior quality.[8] Therefore, the regenerative capacity of pyramidal neurons is probably superior in our coculture system when compared to adult tissues.

■ Conclusion

Previous *in vitro* experiments on axonal sprouting have often relied on explants from cortex or sympathetic ganglions cultured on cryostat sections of brain or spinal cord.[18,19] In other cases, living substrates were used to study regenerative processes such as the extension of neurites from sensorimotor cortex into spinal cord gray matter.[20] We have previously used various fluorescent dyes to visualize fiber tracts in the entorhinal-hippocampal system *in vitro*,[21,22] and we relied on these experimental techniques to establish an *in vitro* slice coculture model that enables studies on the regenerating corticospinal system. The effects of exogenous administration of neurotrophic or antirepellent factors on neuronal regeneration in the corticospinal system can be investigated rather comfortably, as neuronal survival, axonal regeneration, and sprouting all occur in our model. The model has the advantage of organotypic slice cultures, that is, easy access where pharmacological or surgical intervention is intended. Despite of the limitations discussed above, the model should have the potential to serve as a useful tool in experiments that aim at elucidating the neuronal preservation, axonal

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regeneration, and sprouting after various pharmacological interventions.

■ Key Points

- The coculture of slices of cortical and spinal cord tissue is possible and can be used in order to study processes of axonal sprouting.
- The improved survival of cortical neurons in the NT-3-treated group indicates a potent neuroprotective effect of NT-3.
- The presence of white matter inhibits axonal regrowth even in the presence of neurotrophic factors, whereas neurotrophin-induced axonal sprouting can take place when white matter has been eliminated.

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