AUS DER ORTHOPÄDISCHEN UNIVERSITÄTSKLINIK
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Ärztlicher Direktor: Prof. Dr. med. L. Zichner

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Characterization of postnatal rat spinal cord slice cultures and studies on co-cultures of postnatal rat spinal cord and motorcortex

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Dekan:	Prof. Dr. Josef Pfeilschifter
Referent:	PD Dr. N. P. Hailer
Koreferent:	Prof. Dr. T. Deller

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Dedicated to

My father,

My mother,

My sister

And my wife

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1. INTRODUCTION

1.1. SPINAL CORD INJURY (SCI)

1.1.1. Epidemiology and demographics of SCI

Spinal cord injury (SCI) remains a devastating injury for both patients and their families. Recent literature reviews reveal that the reported SCI incidence and prevalence have not changed substantially over the past 30 years.

The annual incidence varies widely among countries and ranges from 10.4 to 83 per million. The mean age at the time of injury is 33 years, and the men/women ratio is reported to be 3.8/1. One third of patients with SCI are tetraplegic and 50% of patients with SCI have a complete lesion (Wyndaele et al, 2006)

On a global level, traffic accidents involving motor vehicles, bicycles or pedestrians account for the greatest number of SCI (almost 50%). Sports-related and recreational injuries including football, riding, surfing, diving, parachuting, rock climbing and skiing have increased, in some countries exceeding work-related injuries. The latter have decreased in many countries, as a result of improved safety in working environment.

While forty years ago approximately two thirds of SCIs were complete, in more recent times only 50% are complete. This changing trend could be attributed to improved initial care and retrieval systems, greater awareness of the importance of immobilization after injury, use of restraints and air bags in motor vehicles, and hospital maneuvers to limit secondary injury, including avoidance of systemic hypotension and hypoxia.

Isolated SCI is relatively rare, as it occurs only in 20% of the cases, while 20-57% of patients suffering from SCI also have significant other injuries such as traumatic brain injury, major thoracic injuries, abdominal injuries, and pelvic or extremity fractures. Since these additional injuries more commonly precipitate hypoxia and hypotension, both of which may cause additional secondary damage to the spinal cord, it has been suggested that these additional injuries have a cumulative effect on reducing the neurologic recovery and worsening the outcome from the SCI, both in terms of mortality and morbidity.

Although it was initially believed that renal failure and other urinary tract infections were the leading causes of death in patients with SCI, more recent studies suggest that respiratory complications are responsible for the majority of deaths. Aside from pneumonia, leading death causes include heart disease, septicemia, pulmonary emboli, suicide and unintentional injuries (Sekhon et al, 2001).

1.1.2. Pathophysiology of SCI

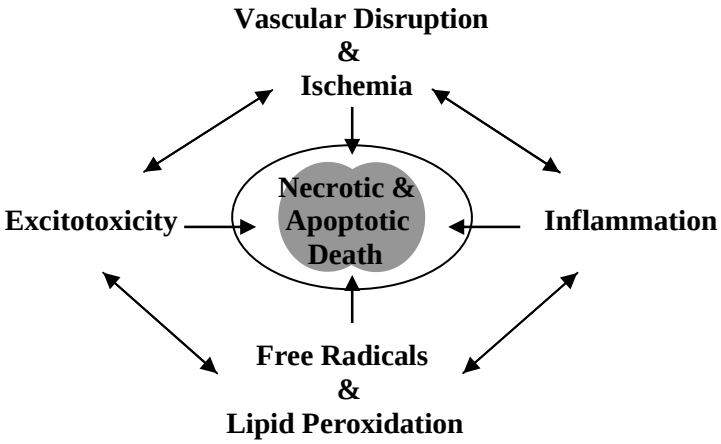
It is now generally accepted that acute SCI is a complex two step lesion involving mechanisms of primary and secondary damage (Kwon et al, 2004).

1.1.2.1. Primary lesion

The primary lesion is defined as the immediate mechanical damage caused to the spinal cord at the time of injury. It is most commonly a combination of the initial impact, as well as subsequent persisting compression, typically occurring with fracture-dislocations, burst fractures and acutely ruptured discs. If the forces are of sufficient magnitude to overcome the resistance of the spinal osteoligamentous structures, the energy is transmitted to the spinal cord. These forces may result in contusion, haemorrhage, shear or laceration of the spinal cord.

1.1.2.2. Secondary lesion

The primary lesion is followed by a complex sequence of cellular responses, finally leading to the death of neurons and oligodendrocytes that were initially spared during the phase of primary damage. The primary mechanical injury initiates a cascade of secondary injury mechanisms that severely -and often lethally- impair the cellular homeostatic mechanisms, thus contributing to the necrotic and apoptotic neuronal cell death after SCI. These interrelated processes enhance the amount of initial injury (Kwon et al, 2004).



Submicroscopically, the impairment of homeostatic mechanisms includes the following: 1) ionic derangements, such as increased intracellular calcium, increased extracellular potassium and increased sodium permeability; 2) neurotransmitter accumulation, including serotonin or catecholamines and extracellular glutamate, which causes excitotoxic cell injury; 3) arachidonic acid release and free radical production, eicosanoid production and lipid peroxidation; 4) loss of adenosine triphosphate-dependent cellular processes, all finally

causing programmed cell death or apoptosis. This cascade results in a severe impairment of neuronal homeostatic mechanisms, axonal degeneration and demyelination (Tzeng et al, 2001).

Vascular mechanisms, free radicals and more recently apoptosis, have received much attention in the effort to elucidate the complex pathophysiology of the secondary lesion after SCI in order to develop possible effective therapies.

1.1.2.2.1. Vascular mechanisms

Changes in spinal cord blood flow play an important role in the secondary damage and can be divided into systemic and local. Immediately after SCI a major reduction in blood flow at the lesion occurs. If left untreated, this ischemia worsens progressively over the first few hours, it may persist for at least 24 h and its effects can be exacerbated through haemorrhage, thrombosis and edema. After an acute SCI, profound hypotension may occur, thus rendering acute SCI one of the causes of neurogenic shock.

On a microscopical level, the vascular damage implicated in the secondary lesion of acute SCI, includes loss of the microcirculation, direct disruption of small vessels and haemorrhage.

1.1.2.2.2. Free radicals

Free radicals, most commonly oxygen free radicals, appear early in the course of secondary lesion and, if unchecked, can cause progressive lipid peroxidation in cellular membranes and consequently more free radicals that can propagate the reaction across the cellular surface. This oxidative stress can also disable mitochondrial respiratory function, alter DNA and DNA-associated proteins and disrupt ionic gradients, collectively conspiring to induce metabolic collapse and subsequent necrotic or apoptotic cell death.

1.1.2.2.3. Apoptosis

Apoptosis is a form of programmed cell death, characterized by cell shrinkage, chromatin aggregation and nuclear pyknosis. It is a tightly regulated process with a sequence of activation steps requiring energy in the form of ATP and *de novo* protein synthesis in order to be completed. These pathways ultimately lead to the activation of caspases, a family of cysteine proteases, which then target various cytosceletal and nuclear proteins to affect an orderly dismantling of the cell.

Recent evidence suggests that apoptosis may be important in the pathophysiology of SCI. Apoptosis occurs in populations of neurons, oligodendrocytes, microglia, and, perhaps, astrocytes. Apoptosis may occur early in neurons at the injury site, while death of oligodendrocytes in white matter tracts continues for many weeks after injury, even at a remote distance from the initial injury site and contributes to post-injury demyelination. Microglial activation seems to be involved in this process (Beattie et al, 2000; Kwon et al, 2004; Sekhon et al, 2001).

1.1.3. Therapeutic interventions in SCI

Since the primary injury is irreversible, much research has focused on the secondary injury, in an effort to develop possible effective therapeutic interventions.

Major advances in the understanding of primary and secondary injury mechanisms have led to the preclinical study of many promising pharmacological therapies, all with the goal of improving neurological outcome. A few of these drugs have stood the test of animal model experiments and have been investigated in human clinical trials.

Methylprednisolone has enjoyed widespread use in the setting of acute SCI since the publication of the preliminary NASCIS II results in 1990. Well documented adverse side-effects including severe pneumonia, sepsis and death were associated with steroid therapy after acute SCI. These side-effects combined with the inability to demonstrate reproducible, long-lasting, significant improvement after steroid treatment for SCI have led to a lively debate in the literature over whether or not steroids should be used in SCI (Hurlbert, 2006).

The AANS/CNS Joint Section on Disorders of the Spine and Peripheral Nerves Guidelines committee reviewed all available evidence and concluded that "…methylprednisolone for either 24 or 48 hours is… an option in the treatment of patients with acute SCI that should be undertaken only with the knowledge that the evidence suggesting harmful side effects is more consistent than any suggestion of 'clinical benefit'." Nowadays, methylprednisolone administration is still practiced in many centers, in most cases because of peer pressure or for fear of litigation (Hurlbert, 2006).

The only other medication to be formally tested through clinical trials for use in SCI is GM-1 ganglioside, which is thought to have anti-excitotoxic activity, promote neuritic sprouting, potentiate the effects of nerve growth factor (NGF) and prevent apoptosis. Initial, very promising results for functional recovery could not be reproduced and consequently, GM-1 ganglioside is not used in routine clinical practice for SCI treatment.

Transplantation of progenitor or stem cells into the injured spinal cord continues to be a promising future therapeutic intervention. After many animal experiments with encouraging results, human embryonic stem cell transplantation in patients with SCI is expected to be performed in the near future.

Neuroprotective strategies aiming at preventing additional neuronal and glial loss caused by secondary injury are intensively studied. These include various monoclonal antibodies, modulation of chemocine release, neurotrophin delivery and inhibition of the GTPase ras homology protein (Rho).

The most important principles in the medical management of patients with acute SCI are oxygenation, blood pressure support through volume replacement (and if necessary inotropes), and immobilization. It is well established that hypoxia, ischemia and hypotension aggravate SCI. It seems that early volume resuscitation and blood pressure augmentation optimize the potential for neurologic recovery. Existing SCI treatment guidelines recommend

avoidance of systolic blood pressure <90 mm Hg and maintenance of mean arterial blood pressure between 85 and 90 mmHg for the first week in patients with acute injury (Hurlbert, 2006).

Surgical intervention in the treatment of acute SCI is an area of intensive research and controversy. There is conflicting evidence, whether early decompression following SCI conveys a benefit in neurological outcome. Animal studies consistently show that neurological recovery is enhanced by early decompression. There was one randomized controlled trial that showed no benefit to early (<72 hours) decompression. Several recent prospective series suggest that early decompression (<72 hours) can be performed safely and may improve neurological outcomes. A recent systematic review showed that early decompression (<24 hours) resulted in statistically better outcomes compared to both delayed decompression and conservative treatment (Fehlings et al, 2006).

Currently, there are no standards regarding the role and timing of decompression in acute SCI. Urgent decompression of bilateral locked facets in a patient with incomplete tetraplegia or in a patient with SCI with neurological deterioration is recommended. Urgent decompression in acute cervical SCI remains a reasonable practice option and can be performed safely. There is emerging evidence that surgery within 24 hours may reduce length of intensive care unit stay and reduce post-injury medical complications (Fehlings et al, 2006).

1.2. CNS REGENERATIVE CAPACITY

Although it has been shown that some neurons proliferate even in the adult CNS, it is widely believed that the capacity of CNS neurons for axonal regrowth and sprouting decreases as the age at the time of injury increases. While after spinal cord lesions at birth there is extensive axonal regenerative growth, axonal regrowth after lesions in the adult CNS is restricted (Bregman et al, 1989; Bregman et al, 1997).

Sprouting is maximal after lesions up to postnatal day 5(p5) and then gradually declines until very little sprouting is seen after lesions performed up to the second postnatal week. After unilateral spinal cord denervation by an early corticospinal tract (CST) lesion, sprouting from the intact corticospinal pathway into the contralateral denervated spinal cord occurred only at local spinal cord levels and not at the pyramidal decussation. Sprouting corticospinal axons arborized with the same degree of functional and topographic specificity as normal corticospinal arbors, such that axons arising from the motorcortex innervated only the ventral horn and normal forelimb and hindlimb topography was preserved (Kuang et al, 1990).

Furthermore, this time-dependent decline in regenerative sprouting is well correlated with the increasing myelination in the spinal cord gray matter. In the rat, immunoreactivity for myelin antigens is limited to some fibre tracts at p5. Only by p16 does the gray matter show

substantial myelination and expression of myelin-associated neurite growth inhibitors, and by p28 the myelination pattern in gray and white matter is rather complete in the rat spinal cord (Vanek et al, 1998).

Myelin seems to be a major source of inhibition of axonal regeneration and sprouting. It was found that a monoclonal antibody (IN-1) generated against a central myelin antigen improved neuronal regeneration. Myelin associated glycoprotein (MAG) has been shown to be a myelin-associated growth inhibitory molecule. Recently, a major achievement was the cloning and expression of a novel protein, Nogo-A which is the specific target of IN-1. Since only a small portion of Nogo-A is expressed on the surface, the cells may need to be damaged for significant quantities of Nogo-A to be released (Behar et al, 2000). Moreover, growth-associated protein 43 (GAP-43) was found to be highly expressed during rat CST development, and high levels of this growth-associated protein are correlated with neurite outgrowth and sprouting throughout this developmental phase. In the myelin-free spinal cord GAP-43 expression was found to be strongly increased.

Taken together, the acquired inability of the CNS to regenerate is mainly attributed to two factors: 1) the presence of an environment hostile to growth (e.g. appearance of myelin-associated inhibitors of axonal growth, alterations in astrocytes and extracellular matrix molecules restricting growth) and 2) the lack of adequate trophic support (e.g. decrease in the expression of growth-associated proteins) (Bregman et al, 1997; Joosten et al, 1995).

In recent years it has become clear that the CNS has the capacity to regenerate under certain conditions, and much research has been focused on stem cells and their possible role in regenerative processes in the adult CNS. Adult corticospinal tract neurons in culture were found to be capable of DNA synthesis and it was suggested that at least a portion of these cells may undergo cell division(Alexanian et al, 2001; Joosten, 1997) .

1.3. NEUROTROPHINS

In an effort to overcome these limiting factors, much attention has been directed on promoting axonal regeneration and sprouting by exogenous administration of neurotrophins. Neurotrophins, a small group of dimeric proteins comprise a family of closely related neurotrophic factors expressed in target tissues of neurons and in the central and peripheral nervous systems.

Neurotrophic factors are proteins that regulate neuronal survival, differentiation, axonal outgrowth, synaptic plasticity and neurotransmission (Lu et al, 2001; Tucker et al, 2001) by activating different intracellular signaling pathways and by augmenting the production of other trophic factors (Kruttgen et al, 1998).

Four major neurotrophins have been identified in rodents: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin 4/5 (NT-4/5).

The biological effects of these factors are mediated by a member of the tumor necrosis factor-α (TNF-α) family of receptors, the low-affinity common neurotrophin receptor $p75^{NTR}$, and the high-affinity tyrosine kinase (trk) family receptors, with NGF binding to TrkA, BDNF and NT4/5 binding to TrkB, and NT-3 binding to TrkC (Barbacid, 1995). At high concentrations, NT-3 can also stimulate Trk-A and Trk-B as nonpreffered receptors (Ryden et al, 1996).

The ability of neurotrophins to activate different intracellular signaling pathways raises the possibility that functional divergence could exist *in vivo*: neurotrophins may separately promote cell survival and axonal outgrowth and may therefore exert one of these biological effects but not the other in a given biological setting (Lu et al, 2001).

Although promotion of neuronal survival is one of the most prominent physiological functions of the neurotrophins, they can even induce neuronal death in developing neurons, depending on which receptor they activate. In this concept, Trk receptors mediate survival signals, whereas p75 mediates the death signal in the absence of the respective Trk receptor (Giehl et al, 2001). Experiments with mice carrying mutations in the NGF and $p75^{NTR}$ genes indicated that a sizeable proportion of the early cell death observed in the developing retina and in the spinal cord was mediated by NGF acting through $p75^{NTR}$. Such mechanisms seem to be used to remove cells and generate space for axonal tract elongation in neuroepithelial developmental phase (Frade et al, 1999). In animals lacking functional neurotrophin or receptor genes, neuronal numbers do not seem to be massively reduced in the CNS during the normally occurring phase of developmental cell death (Lewin et al, 1996).

1.3.1. Neurotrophic and neuroprotective effects of NT-3

Experimental evidence suggests that neurotrophins may prove to be effective in the treatment of SCI. The application of NT-3 after spinal cord lesions stimulated regenerative growth of corticospinal tract (CST) axons and also resulted in increased local sprouting at the lesion site (Grill et al, 1997; Houweling et al, 1998; Schnell et al, 1994). NT-3 was found to upregulate the expression of molecules, such as GAP-43 and the neuron specific T alpha 1 alpha-tubulin, which are associated with axonal growth (Mohiuddin et al, 1995). Moreover, NT-3 demonstrates a neuroprotective effect, since it prevents axotomy-induced cell death and supports post-traumatic neuronal survival (Bradbury et al, 1998; Giehl et al, 1996; Himes et al, 2001; Novikova et al, 2002).

NT-3 has been frequently used in experiments to induce growth of damaged CST axons, since it is expressed in the unlesioned and lesioned cortex and virtually all unlesioned and lesioned corticospinal neurons (CSNs) express the TrkC neurotrophin receptor at any time point, with this expression mainly localized to pyramidal cells of cortical layer V (Giehl et al, 2001).

Apart from its effect on neural cells, NT-3, as other neurotrophins, seems to play a role in the regulation of glial development, proliferation and activation in the CNS. The presence of functional trkA and trkC receptors in developing oligodendrocytes and the strong Trk

upregulation in activated astrocytes indicate an important role for NT-3 on cells of glial origin (Cohen et al, 1996; Wang et al, 1998). In NT-3 and trkC knockout animals, deficiencies in CNS glial cells, including oligodendrocytes, astrocytes and ameboid microglia were observed, supporting an NT-3-dependent mechanism for the normal development of CNS glial cells (Kahn et al, 1999).

Neurotrophins have been shown to induce oligodendrogliagenesis *in vitro*, while in an adult rat spinal cord contusion model NT-3 induced myelinogenesis and proliferation of oligodendrocytes lineage cells (McTigue et al, 1998). NGF, BDNF and NT-3 mRNAs were found in oligodendrocytes *in vivo* and *in vitro*, supporting a novel role for oligodendrocytes in providing local trophic support for neurons in the CNS, particularly during development (Dai et al, 2003).

Similarly, astrocytes are suggested to be local sources of neurotrophins for adjacent neurons, especially during development (Wu et al, 2004). After transient ischemia in the rat spinal cord the expression of various neurotrophins was upregulated not only in neurons, but also in astrocytes (Tokumine et al, 2003).

Furthermore, microglial/macrophage cells express neurotrophins *in vitro* and *in vivo*, suggesting that these cells promote development and normal function of neurons and glia. Developmental regulation of microglial NT-3 expression occurs, with p10 brain microglial cells exhibiting NT-3 immunoreactivity, in contrast to adult brain microglial cells. Microglia were also found to be responsive to neurotrophins, where NT-3 promoted proliferation and induced phagocytic activity (Elkabes et al, 1996).

1.4. EXPERIMENTAL MODELS

1.4.1. Organotypic slice cultures (OSCs)

In recent years OSCs of the hippocampus or the cerebellum (Gahwiler, 1984; Notterpek et al, 1993; Tauer et al, 1996) have become a well characterized *in vitro* model to study neuronal tissue. Organotypic hippocampal slice cultures (OHSCs) have been widely used to investigate neurons and glial cells *in vitro*, including the interaction of these cells with each other (Frotscher et al, 1995; Hailer et al, 1999; Hailer et al, 2001; Heimrich et al, 1993). Organotypic slice cultures of embryonic spinal cord tissue have been successfully maintained up to 2 weeks (Tscherter et al, 2001; Wetts et al, 1998). Models of organotypic spinal cord cultures of postnatal rats (Delfs et al, 1989; Marsh et al, 2000) and adult mice (Krassioukov et al, 2002) have been used but the fate of different cellular populations over longer culture periods has previously not been described.

It is well established that neuronal as well as non-neuronal cells are well preserved in OSCs. Neurons in OHSCs are known to preserve morphological and physiological features of the *in*

vivo situation, while microglial cells in OHSCs are initially activated following explantation but return to a resting state after at least 6 div (Hailer et al, 1996).

In some aspects, organotypic slice cultures are superior to *in vivo* models, as they combine organotypic tissue preservation with the advantage of easy visualization and free access when pharmacological intervention is intended. Cells within OSCs maintain the tissue-specific cell connections that exist *in vivo*, as well as local neuronal circuits with the appropriate patterns of innervation. Furthermore, the more complex parameters, which complicate studies *in vivo*, such as blood supply, blood–brain barrier and invasion of hematogenous cells are excluded in OSCs.

The combination of pharmacological accessibility and long-term survival along with preservation of regional differentiation, neuroanatomical organization and cell-to-cell interaction renders organotypic slice cultures a very useful model in the study of neuronal tissue (Bernaudin et al, 1998).

1.4.2. The rat corticospinal tract (CST)

Several neuronal projections continue to develop postnatally for up to two weeks, such as the corticospinal tract and the rubrospinal tract (Kudo et al, 1993).The rat CST is often used as a model in developmental and regeneration studies because of its well defined anatomical and functional properties, its experimental accessibility, its relevance to human SCI and its postnatal outgrowth throughout the spinal cord.

The rat CST connects discrete regions of the motorcortex to topographically matching targets in the spinal cord. It originates in the primary motorcortex and projects to the spinal cord through a crossed dorsal component that contains 95% of the descending axons and an ipsilateral ventral component containing less than 5% of all CST axons. Two other minor components of the CST also exist, the lateral and dorsolateral components, together constituting less than 2% of CST axons (Weidner et al, 2001).

In rodents corticospinal outgrowth and connectivity develop during the first postnatal weeks. Efferent axons from pyramidal neurons in cortical layer V enter the spinal cord white matter at postnatal day 1 (p1) and after two to three days the first CST axons innervate the spinal gray matter. The rat CST development is characterized by a staggered mode of outgrowth: a main wave of CST fibers is preceded by a number of pathfinding axons, which are characterized by dilations at their distal ends: the growth cones. The CST axons innervate spinal target sites by collateral branches that develop *de novo* from the primary axons (Joosten et al, 1995; Joosten, 1997; Kuang et al, 1994).

It is known that the capacity of the CST for regeneration after injury decreases as the age of the animal at time of injury increases. Lesions of the CST at birth are followed by the aberrant rerouting of the developing corticospinal axons around the lesion site through adjacent undamaged CNS tissue. This developmental plasticity becomes severely restricted by 5-6

days of age, when axons are no longer capable of growth around the site of injury (Bregman et al, 1989).

1.4.3. Axonal labeling

In order to investigate and study the diverse neuronal pathways many methods of anterograde and retrograde neuronal labeling have been described. *In vitro*, tracing with Mini-Ruby, a 10kD biotinylated dextran amine, was effective in an OHSC model, as perforant path axons were successfully traced after Mini-Ruby application on the entorhinal cortex (Kluge et al, 1998). *In vivo*, biotinylated dextran amine has been used successfully in numerous experiments to trace various neuronal pathways. The mechanism of the hydrophilic BDA molecule uptake is likely to be endocytotic for intact neurons, but BDA and dextran amines in general are also taken up by damaged neurons, possibly by diffusion (Reiner et al, 2000).
A further advantage of biotinylated dextran amines is that it is easy to perform a well-defined application in order to confine it to the region of interest. Moreover, it readily tolerates glutaraldehyde fixation with no significant attenuation of labeling, and it only requires a one-step ABC procedure to be visualized (Reiner et al, 2000).

1.5. IMMUNOHISTOCHEMICAL MARKERS

In order to identify and to distinguish neurons, astrocytes, microglial cells, and precursor cells we used well established immunohistochemical markers:

1.5.1. NeuN

NeuN (**Neu**ronal **N**uclei) is a neuronal nuclear protein of 46-48 kDa apparently specific to neurons. Almost all neurons in the CNS and peripheral ganglia, including cerebellum, cerebral cortex, hippocampus, thalamus, spinal cord, dorsal root ganglia, sympathetic chain ganglia and enteric ganglia possess NeuN-immunoreactivity. The few cell types lacking NeuN-immunoreactivity are cerebellar Purkinje cells, mitral cells of the olfactory bulb and retinal photoreceptors.
The immunohistochemical staining is primarily limited to the nucleus of the neurons with lighter staining in the cytoplasm. Developmentally, immunoreactivity is first observed shortly after neurons have become postmitotic, no staining has been observed in proliferative zones. This distribution together with the finding that glial cells and ependymal cells lack NeuN immunoreactivity makes NeuN a highly sensitive and specific marker of spinal cord neurons (Manitt et al, 2001; Todd et al, 1998).

1.5.2. ChAT

Choline **A**cetyl**T**ransferase (ChAT) is the enzyme responsible for the biosynthesis of acetylcholine. ChAT, a single-strand globular protein, is presently the most specific marker of cholinergic neurons in the central and peripheral nervous system. In the CNS, cholinergic neurons are quite ubiquitously present and are also found in the anterior horn of the spinal cord. These ventral horn cholinergic neurons represent motoneurons. As ChAT is synthesized in the perikaryon of cholinergic neurons and transported to the nerve terminals, it is present within cell bodies, dendrites, axons and axon terminals (Oda, 1999; Phelps et al, 1984).

1.5.3. Calbindin

Calbindin-D28k (CB), together with calretinin and parvalbumin, is a member of the so called EF-hand family of calcium binding proteins. CB contains six EF-hand domains, four of which bind Ca^{++}. Calcium binding proteins contribute to calcium homeostasis through their capacity to buffer cytosolic Ca^{++} and may also play a neuroprotective role when potentially dangerous increases in intracellular calcium concentration occur (Goodchild et al, 2000; Magnusson et al, 1996). Calcium binding proteins often serve as a useful molecular marker for certain types of neurons, as each of them has been observed in different subsets of neurons (Li et al, 2000). In the spinal cord, certain subsets of spinal interneurons (Antal et al, 1996), mainly in the dorsal horn (Albuquerque et al, 1999), and Renshaw cells in the ventral horn (Alvarez et al, 1999), display CB-immunoreactivity.

During the ontogenesis of the spinal cord, CB-immunoreactivity is mainly observed in the cell somata, first appearing at embryonic days 12-14, while the peak of immunoreactivity, both regarding the number of cells and the staining intensity, occurs during the perinatal period. From p14 on, the number and intensity of the positive cells decreases, the adult levels being reached at p35 (Zhang et al, 1990). Because CB is distributed throughout the cytoplasm of the neurons containing it, immunocytochemistry can result in extensive staining of the dendritic trees, and thus allows the morphology of immunoreactive neurons to be examined.

1.5.4. GFAP

Glial **F**ibrillary **A**cidic **P**rotein (GFAP) is a 50 kDa protein and the principal 8-9 nm intermediate filament in mature astrocytes of the CNS. As a member of the cytoskeletal protein family, GFAP is thought to be important in modulating astrocyte motility and shape by providing structural stability to extensions of astrocytic processes. GFAP is also important

for the maintenance of myelination as well as the normal white matter architecture (Liedtke et al, 1996).
In the CNS of higher vertebrates, astrocytes become reactive following trauma and respond in a typical manner, called astrogliosis, a process characterized by a rapid increase of GFAP-immunoreactivity (Hadley et al, 1997).
Nowadays, GFAP is used as a reliable marker to identify and characterize the reactions of astrocytes to CNS lesions (Kullberg et al, 2001; Morin-Richaud et al, 1998).

1.5.5. IB_4

Griffonia simplicifolia isolectin B_4 (IB_4) is a 114 kDa glycoprotein binding to galactose-containing glycoconjugates on cell membranes. In the CNS, it has a particularly strong affinity for microglial and perivascular cells, thus being a reliable marker for the detection of microglial cells (Streit et al, 1987). The CNS parenchyma harbors a stable population of usually inactive macrophages, the microglial cells, whose phenotype is thought to represent an adaptation to the specialized neural environment. These cells serve major homeostatic and reparative functions, as evidenced by their prompt response to physiological or stress stimuli, as well as by their ability to secrete cytokines and neurotrophic factors and become phagocytic when neurons are damaged (Kreutzberg, 1996). Microglia also play a role in host defense, and are involved in CNS immune surveillance (Aloisi, 2001).

1.5.6. Nestin

Nestin is a large (200 kDa) embryonic intermediate filament protein abundantly produced in the cytoplasm of many multipotent precursor cells. This protein was named nestin because it was found to be specifically expressed in **ne**uroepithelial **st**em cells (Lendahl et al, 1990). It belongs to class III intermediate filaments, that also include vimentin and GFAP, and structurally it is closely related to class IV intermediate filaments, such as neurofilaments and a-internexin.
The expression of nestin in the prenatal and postnatal developing mammalian CNS reflects the differentiation or proliferative state of neural precursors. At the embryonic neurulation stage, multipotential neuroepithelial precursor cells temporarily express nestin. Substitution of nestin protein by vimentin and GFAP or neurofilaments takes place sequentially during the maturation and differentiation of neural precursor cells. Nestin is down-regulated either at the onset of GFAP or neurofilament expression, or during subsequent differentiation of multipotential neural precursor cells into astrocytes or neurons (Kalman et al, 2001)

1.6. HYPOTHESES

A study on strategies promoting neuronal regeneration after SCI is proposed. The ability of neurons to regenerate under certain conditions and the importance of various growth factors have been mentioned above. Although organotypic slice cultures of other CNS regions have been used as experimental models, organotypic slice cultures of postnatal rat spinal cord have not yet been characterized. From these facts the following thoughts emerge:

Hypothesis 1: An organotypic slice culture of postnatal rat spinal cord is a functional in vitro model, where defined neuronal and glial populations remain preserved over longer culture periods.

To test this hypothesis we investigated the fate of distinct neuronal and glial populations using well established immunohistochemical markers.

Hypothesis 1a: Tissue preservation of spinal cord OSCs is influenced by two important parameters:

i) the animal age at the time of explantation, and

ii) the duration of the in vitro period.

Hypothesis 1b: In a SCSC model there is unavoidable interruption of all afferent and efferent neuronal pathways. Degenerative changes in defined neuronal populations and glial activation are therefore to be expected.

To address these questions we studied OSCs derived from animals of different ages and sustained *in vitro* over different time periods, and characterized them by immunohistochemistry.

Having established and characterized the postnatal rat SCSC model, we investigated the possibility of establishing a co-culture model of postnatal rat motorcortex and spinal cord slice cultures, where the effects of the exogenous administration of neurotrophic factors on neuronal survival and neuronal circuitries regeneration after experimental lesions could be investigated.

SCI causes a disruption of CST. While NT-3 and other neurotrophic factors can induce regrowth of CST axons *in vitro* and *in vivo*, it is known that white matter inhibits axonal regeneration and sprouting. These facts lead to following hypothesis:

Hypothesis 2: Positioning of cerebral cortex adjacent to spinal cord explants should enable outgrowth of CST axons and enable studies on NT-3 effects.

Hypothesis 2a: Exogenous NT-3 administration has the potential to improve neuronal survival and to promote axonal regrowth in vitro.

Using anterograde labeling of sprouting corticospinal axons, two groups were examined: one control group and one group receiving NT-3.

Hypothesis 2b: Spinal cord white matter inhibits axonal outgrowth and penetration into spinal cord gray matter.

To investigate this, we performed experiments using two different subgroups: one with interposition of spinal cord white matter between cortical and spinal gray matter, and one without white matter interposition.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Substances, solutions and antibodies

The following substances, solutions and antibodies were used in the experiments:

Agar (Merck, Darmstadt, Germany)

Antibodies:

- -Goat anti-rabbit IgG biotin-conjugated (Sigma Chemicals, Deisenhofen, Germany/ cat. no: B8895)
- -Mouse anti-Choline Acetyltransferase (ChAT) monoclonal antibody (Chemicon, Temecula, CA, USA / cat. no:MAB305)
- -Mouse anti-Glial Fibrillary Acidic Protein (GFAP) monoclonal antibody (Chemicon/ cat. no:MAB360)
- -Mouse anti-Nestin monoclonal antibody (Chemicon/ cat. no:MAB353)
- -Mouse anti-Neuronal Nuclei (NeuN) monoclonal antibody (Chemicon/ cat. no:MAB377)
- -Rabbit anti-Calbindin D-28K polyclonal antibody (Chemicon /cat. no: AB1778)
- -Rabbit anti-mouse IgG biotin-conjugated (Sigma/ cat.no: B8520)

Bovine serum albumin (BSA, Sigma)

Calcium dichloride -Dihydrate ($CaCl_2$*$2H_2O$; Merck)

Cell cultures inserts (Becton Dickinson, Heidelberg, Germany; pore size 0.4 µm)

6-well culture dishes (Becton Dickinson)

Dextran amine 10kD biotinylated (MiniRuby; Molecular Probes, Eugene, OR, USA)

3, 3´-Diaminobenzidine (DAB; Sigma)

Entellan (Merck)

Ethanol (Mallinckrodt Baker, Deventer, The Netherlands)

ExtrAvidin Peroxidase (Sigma)

Glucose (Braun, Melsungen, Germany)

Glutamine (Gibco BRL Life Technologies, Eggenstein, Germany)

Glutaraldehyde (Sigma)

Griffonia simplicifolia Isolectin B_4 biotin-conjugated (IB_4; Sigma/ cat. no: L2140)

Hank's balanced salt solution (HBSS; Gibco)

Hematoxylin (AppliChem, Darmstadt, Germany)

Histoacryl tissue glue (Braun)

Hydrochloric acid 25% (Merck)

Hydrogen peroxide (H_2O_2; Merck)
Insulin (Boehringer, Mannheim, Germany)
Magnesiumsulfate-Heptahydrate ($MgSO_4*7H_2O$; Merck)
Methanol (Merck)
Minimal essential medium (MEM, Gibco)
Normal horse serum (NHS; Gibco)
NT-3 (rhNT-3, R&D Systems, Wiesbaden, Germany)
Paraformaldehyde (Merck)
Penicillin (Sigma)
Picric acid, saturated (Merck)
Potassium chloride (KCl; Merck)
Saccharose (Merck)
Sodium azide (NaN_3; Merck)
Sodium chloride (NaCl; Merck)
Sodium dihydrogen phosphate-Monohydrate ($NaH_2PO_4*H_2O$; Merck)
Sodium hydrogen carbonate ($NaHCO_3$; Merck)
Streptomycin (Sigma)
Tissue-Tek mold release (Sakura Finetek, Zoeterwoude, The Netherlands)
Tris-hydroxymethylaminomethane (THAM; Merck)
Vitamin C (Sigma)
Xylol (Merck)

2.1.2. Storage of substances and antibodies

Glutamine, normal horse serum, insulin, glucose, streptomycin, penicillin and vitamin C were aliquoted and stored at -20°C.
Anti-NeuN, anti-ChAT, anti-Calbindin, anti-GFAP and anti-Nestin antibodies as well as NT-3 and DAB were aliquoted and stored at -20°C.
Biotinylated IB_4 was aliquoted and stored at -80°C.
HBSS, MEM, ExtrAvidin Peroxidase, Hydrogen peroxide, anti-mouse and anti-rabbit antibodies as well as MiniRuby were stored at +4°C.
All other materials were stored at room temperature.

2.1.3. Buffer solutions

The following buffer solutions were used in the experiments:
(Quantities diluted in one litre Aqua destillata [dest.]):

Phosphate buffer (PB) 0,1 M :

11.36 g Na_2HPO_4

2.72 g KH_2PO_4

Phosphate buffer (PB) 0,2 M :

22.72 g Na_2HPO_4

5.44 g KH_2PO_4

Phosphate buffer (PBS) 0,02 M:

9 g NaCl

0.54 g KH_2PO_4

2.27 g Na_2HPO_4

Phosphate buffer (PBS/Triton) 0,02 M mit Triton X:

9 g NaCl

0.54 g KH_2PO_4

2.27 g Na_2HPO_4

3 ml Triton X

Tris-buffer:

6.06 g Tris

All buffer solutions were adjusted to a pH-value between 7.35 and 7.4.

2.1.4. Preparation medium and culture medium

Preparation medium consisted of minimal essential medium (MEM), containing 1% glutamine with a pH-value of 7.35. Throughout the preparation process preparation medium was kept on ice, in order to maintain a temperature of approx. 4°C.
Culture medium consisted of 50% MEM, 25% Hank's balanced salt solution (HBSS), 25% normal horse serum (NHS), 2% glutamine, 1 µg/ml insulin, 2.4 mg/ml glucose, 0.1 mg/ml streptomycin, 100 U/ml penicillin, and 0.8 µg/ml vitamin C. Its pH value was adjusted to 7.4.

2.1.5. Establishment of agar-blocks

In order to establish the required agar-blocks for the slice culture preparation the following ingredients were diluted in 100 ml Aqua dest.:
0.725 g NaCl, 0.0373 g KCl, 0.01725 g $NaH_2PO_4*H_2O$, 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 min to 70°C on a magnetic stirrer and aliquoted in cell culture dishes (35 mm diameter), which, after cooling down, were tightly sealed and stored at 4°C.

2.1.6. Devices

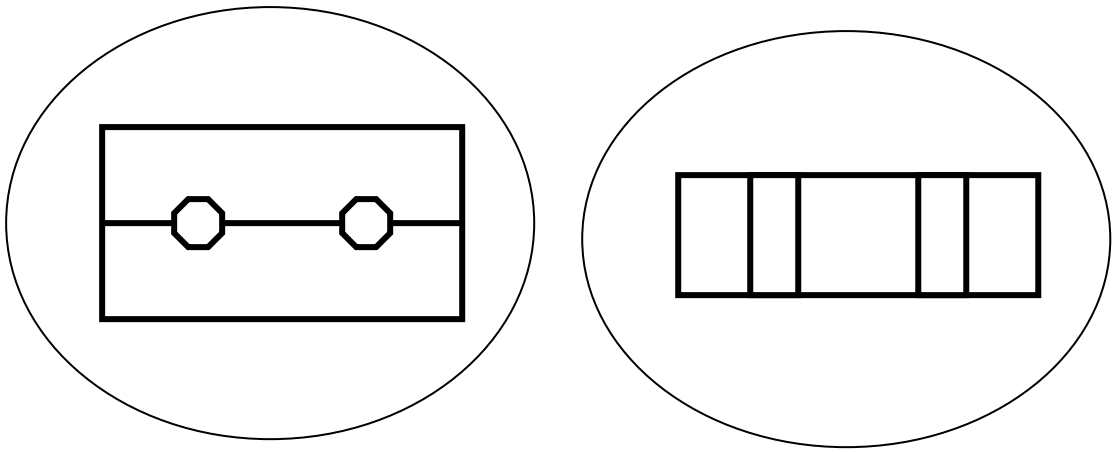
The following devices were used:
Laminar flow workbench (E.R.T. Edelstahl-Reinraum-Technik, type MSW 19)
Sliding vibratome (TPI St. Louis, Missouri, USA, type Vibratome 1000 Classic)
Incubator (Heraeus Instruments, type Heracell)

Light microscope (Leica, Bensheim, Germany, type DM IL)
Laboratory precision scale (Sartorius AG, Göttingen, Germany, type BP221S)
Cryostat (Microm, Walldorf, Germany, type HM 560)

2.2. METHODS

2.2.1. Preparation of rat spinal cord OSCs

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. All experimental manipulations were 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 anatomical forceps, 2 curved anatomical 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, 2 round filters (diameter 55 mm).
Before the preparation, Agar-blocks were prepared as following: An Agar-block measuring approximately 2cm x 1,5cm x 1cm was cut off the above described Agar aliquots and placed on a sterile round filter. Using the tip of the Pasteur pipette 2 holes were made, which measured approximately the diameter of the rat spinal cord. Subsequently, the Agar-block was bisected by using the scalpel blade No 11, with the cutting line passing through the channels opened before (Figure 1a). The one half was placed horizontally, in order to accept the prepared spinal cord segments (Figure 1b).



a **b**

Figure 1: Schematic drawing of Agar-blocks preparation as seen from above (a) and from the side (b).

In order to obtain rat spinal cord organotypic slice cultures, p0 to p12 (p0, p3, p6, p9 and p12) Wistar rats were used.

After decapitation with the large bandage scissors, the dorsal skin and musculature of the trunk were removed along the midline, using the small scissors and the 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 was opened, the spinal cord dissected from the denticulate ligaments and immediately placed in ice cold preparation medium.

Using the anatomical forceps and the No 15 scalpel, the remnants of the surrounding dura matter spinalis were removed under microscopic control with a binocular microscope, and the spinal cord was cut into two segments; one cervicothoracic and one thoracolumbar. Both tissue segments were sandwiched between the two agar half-blocks described above, and the whole block was transported with the curved anatomical forceps onto the Vibratom's Plexiglas-inset, where it was secured with Histoacryl tissue-glue. This construct was reliable in providing 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-inset was filled with ca 50 ml ice cold preparation medium and the spinal cord was cut into transverse slices of 400 µm thickness.

2.2.2. Culture of OSCs

From each spinal cord 10-20 slices were obtained and transferred into cell cultures inserts (pore size 0.4 µm) that were placed in 6-well culture dishes. Two to three slices were placed in the same insert. Each well contained 1 ml culture medium. The culture dishes were incubated at 35°C in a fully humidified atmosphere with 5% CO_2, and the culture medium was changed every second day.

2.2.3. Fixation of OSCs

After zero to twelve days *in vitro* (div) the rat spinal cord OSCs were fixed with a mixture of 4% paraformaldehyde, 15% picric acid and 0.1% glutaraldehyde in 0.2 M phosphate buffer (PB) for 15 min, washed with 0.1 M PB (3x10 min) and postfixed in the same fixative without glutaraldehyde for 1 hr. The fixed OSCs were finally washed with 0.1 M PB for 1 hr, until the yellow color of the picric acid was completely washed away, and were carefully removed from the cell culture membrane using a fine paint brush. The slices were placed in 0.8 M sucrose solution containing 1.5% NaN_3 and stored at 4^{o}C until further processing. In order to prepare the fixed slices for immunohistochemistry, they were sectioned using a

cryostat at -19°C box temperature, with a knife temperature of -22°C. Firstly, the slice cultures were placed on top of a block of frozen Tissue Tek, covered with a drop of Tissue Tek and left to freeze for a few minutes. The entire block was stabilized on a cryostat inset and was sectioned horizontally in the cryostat, thus obtaining transverse slices of 14 µm thickness that were subsequently mounted on gelatine-coated glass slides and stored at -19°C until immunohistochemistry proceeding .

2.2.4. Immunohistochemistry

After pre-treatment with methanol and H_2O_2 (0.45%) in order to achieve endogenous peroxidase quenching, the sections were washed with 0.02M phosphate-buffered saline (PBS) containing 0.3% Triton X-100 (3 x 10 min) and incubated with normal horse serum (NHS, diluted 1:20 in PBS-Triton) for 30 min to block non-specific binding sites.

For immunohistochemical characterization of different cell populations the following primary antibodies were used: 1) monoclonal mouse anti-NeuN, 2) monoclonal mouse anti-ChAT, 3) polyclonal rabbit anti-Calbindin, 4) monoclonal mouse anti-GFAP, 5) monoclonal mouse anti-Nestin, and 6) biotinylated IB_4. SCSCs were incubated with the primary antibody either for 24 hrs (NeuN, ChAT, Calbindin, GFAP, Nestin), or with biotinylated IB_4 for 1 hr, diluted 1:100 (NeuN, ChAT), 1:400 (CB), 1:200 (GFAP), 1:1,000 (Nestin), or 1:20 (IB_4) in 0.02M PBS containing 0.3% Triton with 5% bovine serum albumin (BSA).

The presence of saline in the diluent breaks weak electrostatic forces between non-specifically bound proteins and the addition of Triton X-100, a non-ionic detergent, reduces hydrophobic interactions between tissue and reagent proteins, while BSA serves as an additional source of protein that competitively binds to non-specific binding sites, thereby preventing non-specific binding of the antibodies.

After thorough washing with PBS-Triton (3 x 10 min) the secondary antibody (anti-mouse for NeuN, ChAT, GFAP and nestin, anti-rabbit for CB) was applied for 1 hr, both diluted 1:100 in PBS-Triton. Slices treated with IB_4 were excluded from the application of the secondary antibody, since IB_4 was already biotin-conjugated.

Following repeated washing with PBS (3 x 10 min) preparations were incubated with avidin-biotin-complex (diluted 1:100 in PBS-Triton) for 1 hr and washed with PBS (2 x 10 min) and 0.05M Tris-buffer (pH=7.4; 1 x 10 min).

Subsequently, 3,3´-diaminobenzidine (DAB) was used as a chromogene (0.1 g DAB diluted in 200ml Tris-buffer and activated with 100µl H_2O_2 [40%]). The reaction proceeded under visual control with a light microscope and when coloration was clearly visible, the reaction was stopped with Tris-buffer for 10 min. Finally, the sections were counterstained with hematoxylin, dehydrated through a graded series of ethanol and xylol and coverslipped with Entellan.

2.2.5. Statistical analysis

For statistical analysis the cell number of at least 3 samples from each group was counted using a light microscope. The following groups were included in the statistical analysis:

P-div: age in days-days *in vitro*

n: number of samples, where cell count was performed

v.h.: ventral horn

d.h.: dorsal horn

w.m.: white matter

g.m.: gray matter

NeuN v.h.

P-div	0-0	0-3	0-6	0-9		3-0	3-3	3-6	3-9	3-12
n	4	4	4	3		3	3	4	3	7
P-div	6-0	6-3	6-6	6-9	6-12	9-0	9-3	9-6	9-9	9-12
n	3	3	3	3	4	3	3	3	4	3

NeuN d.h.

P-div	0-0	0-3	0-6	0-9		3-0	3-3	3-6	3-9	3-12
n	4	4	4	3		3	3	4	3	7
P-div	6-0	6-3	6-6	6-9	6-12	9-0	9-3	9-6	9-9	9-12
n	3	3	3	3	4	3	3	3	4	3

Calbindin

P-div	0-0	0-3	0-6	0-9		3-0	3-3	3-6	3-9	3-12
n	3	3	4	4		3	8	4	6	3
P-div	6-0	6-3	6-6	6-9	6-12	9-0	9-3	9-6	9-9	9-12
n	3	3	3	4	4	3	3	3	4	3

GFAP w.m.

P-div	0-0	0-3	0-6	0-9		3-0	3-3	3-6	3-9	3-12
n	3	3	3	3		3	3	4	4	3
P-div	6-0	6-3	6-6	6-9		9-0	9-3	9-6	9-9	
n	5	8	4	5		3	3	3	3	

GFAP g.m.

P-div	0-0	0-3	0-6	0-9		3-0	3-3	3-6	3-9	3-12
n	3	3	3	3		3	3	4	4	3
P-div	6-0	6-3	6-6	6-9	6-12	9-0	9-3	9-6	9-9	
n	5	8	6	5	4	3	3	3	3	

IB_4

P-div	0-0	0-3	0-6	0-9		3-0	3-3	3-6	3-9	3-12
n	3	3	4	6		3	4	5	5	5
P-div	6-0	6-3	6-6	6-9	6-12	9-0	9-3	9-6	9-9	9-12
n	3	5	5	10	3	3	3	5	5	3

Nestin

P-div	0-0	0-3	0-6	0-9		3-0	3-3	3-6	3-9	3-12
n	3	5	3	4		3	3	3	3	3
P-div	6-0	6-3	6-6	6-9	6-12	9-0	9-3	9-6	9-9	9-12
n	3	4	5	3	3	3	3	3	3	3

The mean cell number in each group was calculated and statistical analysis using non-parametric tests was performed due to relatively small sample sizes and skewed data distribution. The one-way ANOVA (ANalysis Of Variance) test combined with Bonferroni's adjustment was used to determine whether there were statistically significant differences in cell numbers among various groups, and $p<0.05$ was considered significant.

2.2.6. Preparation and culture of rat cortex and spinal cord co-cultures

In order to obtain organotypic slice co-cultures of rat cortex and spinal cord p4 Wistar rats were used.

Before the beginning of the preparation procedure, two agar-blocks approximating in dimensions the length and width of the rat brain were fixed onto the Vibratom's Plexiglas-inset with Histoacryl tissue-glue, forming a half rectangular parallelogram (⌐‾) configuration. A third agar-block was added after the positioning of the brain, in order to achieve maximal stabilization during sectioning (⌐¬).The construct was open at the side facing the vibratome's blade.

After decapitation with the large bandage scissors, the scalp was removed using the small scissors and the surgical forceps. Subsequently, by use of the other small scissors and surgical forceps, the calvaria was opened and removed, revealing the underlying brain. With a sharp No 11 scalpel the frontal pole and the cerebellum were removed and the brains were

immediately placed into ice-cold preparation medium. After drying on a filter for a few seconds, the brain was carefully transported with the curved anatomical forceps onto the Vibratom's Plexiglas-inset, where it was secured with Histoacryl tissue-glue and embedded in the agar blocks as described above. After attaching the halved razor blade to the sliding vibratome and filling the Plexiglas-inset with ca 50 ml ice cold preparation medium, 4-6 400μm thick coronal sections were obtained from each brain. Using the anatomical forceps and a No 15 scalpel, the remnants of the surrounding dura matter were removed under microscopic control with a binocular microscope, and the brain slices were transferred into cell cultures inserts that were placed in 6 well culture dishes, containing 1 ml of the above described culture medium per well. The culture dishes were kept in the incubator during the following preparation of the spinal cord slices.

Other animals of the same age were used to obtain transverse spinal cord sections of the same thickness, following the procedure described above. Transverse spinal cord slices were placed under microscopic control adjacent to the coronal brain sections, and the culture dishes were incubated at 35°C in a fully humidified atmosphere with 5% CO_2. 1 hr after preparation, motorcortex pyramidal neurons were anterogradely labeled with MiniRuby, a 10kD biotinylated dextran amine that is anterogradely transported in intact axons. The culture dishes were immediately returned to the incubator, and the culture medium was changed every second day.

2.2.7. Subgroup division

The cultures were divided into two groups: The NT-3 group, where the culture medium contained 50 ng/ml NT-3 (rhNT-3), and the control group treated with normal culture medium as described above. Each group was further divided into two subgroups: In the first subgroup, intact spinal cord slices cultured adjacent to motorcortex slices were used ("whole slice" subgroup), while in the second subgroup the spinal cord slices were sagitally cut into halves under microscopic control, with the sectioned interface placed directly adjacent to the motorcortex ("half slice" subgroup). The latter protocol was used in order to prevent the spinal white matter from interfering between the cortical and spinal gray matters (Figure 2).

2.2.8. Fixation of rat cortex and spinal cord co-cultures

After 4 days *in vitro* (div) the cocultures were fixed with 4% paraformaldehyde, 15% picric acid and 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB) for 15 min, washed with 0.1 M PB and postfixed in the same fixative without glutaraldehyde for 1 hr. Cocultures were finally washed with 0.1 M PB for 1 hr, until the yellow color of the picric acid was completely washed out, and were carefully removed from the cell culture membrane using a fine paint

brush. The slices were placed in 0.8 M sucrose solution containing 1.5% NaN_3 and were stored at 4°C.

2.2.9. Immunohistochemistry

After pre-treatment with methanol and H_2O_2 (0.45%), the co-cultures were washed with phosphate-buffered saline (PBS) and incubated with avidin-biotin-complex (diluted 1:100 in PBS with 0.3% Triton) for 1hr. Following thorough washing with PBS (2 x 10 min) and Tris-buffer (1x10 min), 3, 3´-diaminobenzidine (DAB) was used as a chromogene (0.1 g DAB diluted in 200ml Tris-buffer and activated with 100µl H_2O_2 [40%]). The reaction proceeded under visual control with a light microscope and when coloration was clearly visible, the reaction was stopped with Tris-buffer for 10 min. After washing, co-cultures were mounted on gelatine-coated glass slides, counterstained with hematoxylin, dehydrated through a graded series of ethanol and xylol and coverslipped with Entellan.

Figure 2:

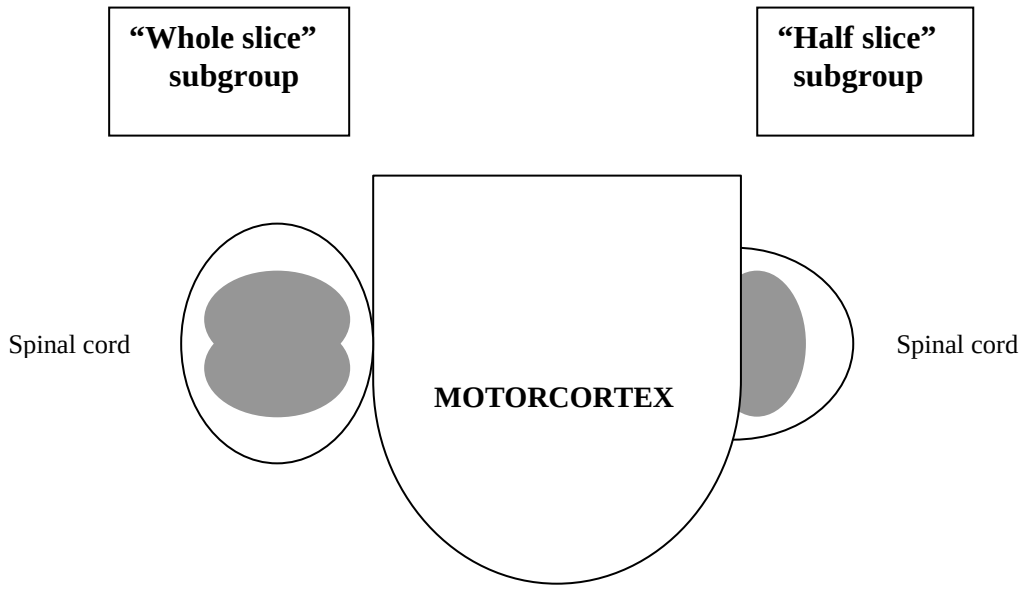


Figure 2: Schematic drawing of motorcortex and spinal cord slice cultures and their orientation in the a) "whole slice" and b) "half slice" subgroups.

3. RESULTS

3.1. PART I: CHARACTERIZATION OF SPINAL CORD SLICE CULTURES (SCSCs)

3.1.1. Distribution of neuronal and glial cell populations in the spinal cord (description of acutely fixed slices)

The spinal gray matter is divided into 10 cytoarchitectonic regions, laminae I-IX (from dorsal horn to ventral horn) and an area around the central canal (area X) (Paxinos et al, 1986; Rexed, 1952; Rexed, 1954) .

Comparing acutely fixed slices, the obvious macroscopically visible difference between slices from the various age groups was in the size of the slice, as the diameter of the spinal cord increases with the age of the animal. In contrast, the immunohistochemical aspect of the acutely fixed preparations revealed no significant differences among various age groups, with the exception of nestin-immunoreactivity: The nestin-immunoreactivity in acutely fixed slices was influenced by the age of the animal, as described below in detail. The following description of NeuN-, ChAT-, Calbindin-, GFAP- and IB_4-immunoreactivity is representative of acutely fixed slices from all age groups studied.

3.1.1.1. NeuN and ChAT-immunoreactivity (Fig. 3, 4 / p. 31-33)

$NeuN^+$ neurons were distributed across the entire spinal gray matter with almost no immunoreactivity seen in the white matter (Fig. 3a). In the head of the dorsal horn (laminae I, II, III) smaller, round $NeuN^+$ neurons with a compact arrangement prevailed (Fig. 3c). In the neck and the base of the dorsal horn (laminae IV, V, VI) the $NeuN^+$ cells appeared to be more loosely arranged and more heterogeneous in shape and size, with some of them being multipolar and considerably larger. The intermediate zone of the gray matter and the base of the ventral horn (laminae VII, VIII) were generally occupied by loosely arranged neurons of varying size. In the head of the ventral horn, the larger motoneurons could be clearly recognized (Fig. 3b, 4b) occupying lamina IX. The range of their soma sizes indicated that both larger α-motoneurons and smaller γ-motoneurons were present. They displayed strong NeuN-immunoreactivity; this was strongest in the nucleus, weaker in the perikaryal cytoplasm and often extended into the proximal dendrites. These ventral horn motoneurons also demonstrated a strong ChAT-immunoreactivity (Fig. 4d). In the dorsal horn the neurons were smaller and more densely packed than those in the adjacent area (Fig. 3c). Ependymal cells lining the central canal were not $NeuN^+$.

3.1.1.2. Calbindin-immunoreactivity (Fig. 5 / p. 34-35)

Calbindin D28k (CB) was distributed throughout the cytoplasm of neurons, and immunohistochemistry resulted in extensive staining of the dendritic trees that allowed for visualization of the dendritic morphology. In general, CB-immunoreactivity was distributed across the entire gray matter, but was also found in the white matter (Fig. 5a). The head of the dorsal horn (laminae I, II, III) displayed a strong CB-immunoreactivity with densely packed CB^+ structures that represented dendrites and axons, and small round cells, forming a dense CB^+ band in the superficial dorsal horn. CB^+ neurons constituted a morphologically homogeneous population of smaller neurons, some of which gave rise to stained dendrites or processes. In laminae IV, V, VI scattered CB^+ cells were occasionally seen. The ventral horn (laminae VII, VIII, IX) harbored a dense network of CB^+ fibers (Fig. 5b). Many medium to large-sized neurons with long processes were frequently found, many of which could be identified as Renshaw cells (Fig. 5d). Renshaw cells are inhibitory interneurons making contact predominantly on the dendrites of their target motoneurons and are known to be CB-immunoreactive. Large numbers of CB^+ fibers were found in the funiculi of the spinal cord white matter, with the highest density of CB^+ fibers found in the dorsolateral funiculus, while the pyramidal tract did not contain labeled fibers. Ependymal cells lining the central canal displayed a strong CB-immunoreactivity, with the most prominent immunoreactivity being found ventrally and dorsally to the central canal. At the dorsal aspect of the spinal canal, a cluster of CB^+ cell bodies and fibers could be observed, and many of the CB^+ fibers crossed the midline dorsally.

3.1.1.3. GFAP-immunoreactivity (Fig. 6 / p. 36-37)

GFAP immunoreactivity was most prominent in the white matter (Fig. 6a). Astrocytes of the external membrana were strongly stained by the anti-GFAP antibody, and most white matter astrocytes showed the typical radial organization, which was more prominent in the ventral and lateral portions of the white matter. The predominant astrocyte was radially oriented and appeared to span the white matter from the pial surface to the interface between the gray and white matter (Fig. 6b). GFAP-immunoreactivity was barely detectable in the gray matter, where only few $GFAP^+$ astrocytes were visualized, presenting a small stellate perikaryon and a few thin, branched processes (Fig. 6c). Central canal ependymal cells were devoid of GFAP-immunoreactivity.

3.1.1.4. IB_4 –immunoreactivity (Fig. 7 / p. 38-39)

IB_4 staining was very weak in immediately fixed slices (Fig. 7a, b). The most prominent IB_4^+ structures were the remnants of spinal capillaries scattered across all over the spinal cord surface including both the gray and the white matter. A very limited number of IB_4^+ microglial cells without specific spatial distribution were observed. These cells almost exclusively exhibited an amoeboid morphology with round or elongated somata. Only few

cells had developed one or two very small mainly unbranched processes. Virtually no microglial cells that displayed the typical ramified, "resting state" morphology were found.

3.1.1.5. Nestin-immunoreactivity (Fig. 8 / p. 40-41)

The only difference between different age groups observed in acutely fixed slices concerned nestin-immunoreactivity: In slices fixed immediately after explantation and derived from p0 to p6 animals, white matter nestin-immunoreactivity was mainly localized in the ventral and lateral portions (Fig. 8a). The pial surface displayed an increased nestin-immunoreactivity. Long, radially oriented arborising processes ran from this region towards the interface between the white and gray matter (Fig. 8b). The pattern was almost identical to that of GFAP-immunoreactivity seen in the white matter. Some scattered nestin$^+$ elements resembling protoplasmic astrocytes were found in the gray matter. Around the spinal canal increased nestin immunoreactivity was observed. Ependymal cells lining the spinal canal were nestin$^+$ with the most profound signal localized in the ventral and dorsal ependymal cells, from which nestin$^+$ processes were observed to spread towards the gray matter and to reach the ventral and dorsal border of the spinal cord (Fig. 8c). This pattern closely resembled the pattern of the CB-immunoreactivity around the central canal. Labeling of blood vessels was also found in immediately fixed slices but it disappeared with prolonged culture periods. In contrast, in older animals (p9 or p12) nestin-immunoreactivity had a similar spatial distribution, but was significantly less intense, (Fig. 8 j, k, l). Overall, there was a downregulation of nestin-immunoreactivity correlating with increasing animal age.

3.1.2. The spatiotemporal reaction pattern of neuronal and glial cells in SCSCs during culture

Overall, cultures derived from younger animals (p0 or p3) appeared to be better preserved than those derived from older animals (p9 or p12). With prolonged culture time there was a gradual degradation in tissue quality and cytoarchitecture preservation, appearing mainly in the form of vacuolization and being more severe in the outer borders of the white matter. The most prominent alterations, however, occurred during the first three days *in vitro*, with the appearance of the various cellular populations remaining generally unchanged afterwards.

3.1.2.1. Early motoneuron death (Fig. 3, 4 / p. 31-33; Table 1 / p. 42-43)

Already after 3 div the larger motoneurons, originally situated in the ventral horn and displaying a strong NeuN and ChAT-immunoreactivity, (Fig. 4 a-d) began to show signs of degeneration (Fig. 3e and Fig. 4 e-h). The number of the ChAT$^+$ motoneurons was reduced, as was the intensity of the remaining ChAT-immunoreactivity. This was accompanied by a slight decrease in the number of NeuN$^+$ neurons, but many neurons continued to display strong NeuN-immunoreactivity and a healthy morphological phenotype (Fig. 3h). The NeuN$^+$

cells in the dorsal horn appeared to be well preserved, there was no significant reduction in their numbers or alteration of their morphology (Fig. 3f, i). After 6 div ChAT-immunoreactivity in the ventral horn was barely detectable, and NeuN-immunoreactivity was further reduced. After prolonged culture periods (9 to 12 div), the pattern of NeuN-immunoreactivity remained mainly unchanged in younger animals (p0-6): A decrease in the total amount of $NeuN^+$ cells was observed in the ventral horn, while in the dorsal horn the number of $NeuN^+$ neurons remained largely unchanged, although some signs of degeneration could be detected (Fig. 3j-l and Table 1). Longer culture periods (9-12 div) in older animals (p9-12) induced a statistically significant reduction in the number of $NeuN^+$ cells both in the ventral and dorsal horn (Table 1), but a large number of $NeuN^+$ cells remained visible in the dorsal horn (Fig 3. m-o). Taken together, it appeared that the most significant neuronal cell loss occurred in the early culture period and it declined afterwards, with the most prominent feature being the early death of the large ventral horn motoneurons, while other $NeuN^+$ neurons remained well preserved especially in younger animals.

3.1.2.2. Calbindin$^+$ neurons persist in SCSCs (Fig. 5/ p. 34-35; Table 1 / p. 42-43)

After 3-6 div CB-immunoreactivity in the dorsal horn was well preserved (Fig. 5c, e), especially in younger animals. Some of the dorsal horn CB^+ neurons gave rise to stained dendrites or processes. Many large ventral horn CB^+ cells remained intact, showing a healthy morphology and strong CB-immunoreactivity with robust staining of their dendritic trees (Fig. 5d). The dense network of CB^+ fibers also remained clearly visible (Fig. 5f). With prolonged time *in vitro* CB-immunoreactivity appeared to increase in the ependymal cells lining the spinal canal. The cluster of CB^+ cell bodies and fibers, located at the dorsal aspect of the central canal, appeared to expand with prolonged culture periods, and the CB^+ fiber-zone that crossed the midline dorsally became more prominent. Generally, even after prolonged culture periods (9 to 12 div), CB immunoreactivity appeared well preserved in both the gray and the white matter, although a significant reduction in the total number of CB^+ neurons did occur after 9 div in younger animals and after 3-6 div in older animals (Fig. 5g-h and Table 1).

3.1.2.3. GFAP-immunoreactivity in SCSCs increases early during the culture period (Fig. 6 / p. 36-37; Table 1/ p. 42-43)

After 3 div a strong increase in GFAP-immunoreactivity was observed (Fig. 6d). $GFAP^+$ astrocytes, initially restricted almost exclusively to the white matter, were now found in large numbers in the gray matter. In the white matter there was a slight increase in the staining of the processes of the radial cells (Fig. 6e). Although the typical radial organization of the white matter astrocytes was in many cases less apparent, their total number demonstrated no significant alteration throughout longer culture periods (Table 1). The most prominent increase in GFAP-immunoreactivity was observed in the gray matter. Already after 3 div a

statistically significant increase in the number of gray matter $GFAP^+$ astrocytes was observed in both younger and older animals, and it remained elevated for the remaining culture period (Table 1). Gray matter astrocytes appeared tightly packed, hyperfilamentous and displayed thick, richly branched, strongly immunoreactive processes (Fig. 6f). After longer culture periods (6 to 12 div, Fig. 6g-l) GFAP-immunoreactivity remained high in both white (Fig. 6h, k) and gray (Fig. 6i, l) matter without signs of alteration in the morphology of $GFAP^+$ astrocytes.

3.1.2.4. IB_4-immunoreactivity increases in the white matter (Fig. 7 / p. 38-39; Table 1/ p. 42-43)

After 3 div IB_4-immunoreactivity increased mainly in the dorsal tract, in the ventral and lateral portions of the white matter, and at sites of focal tissue destruction (Fig. 7c-f). Two morphologically distinct forms of microglial cells were observed, representing the "resting" and the "activated" state: The former exhibited a ramified morphology, displaying multiple long, richly branched processes, and was found scattered across the entire spinal cord, both in the white and the gray matter (Fig. 7d). The latter phenotype was overwhelmingly found in the white matter and demonstrated large somata with round or amoeboid morphology (Fig. 7f). There was a considerable increase in cell size and staining intensity when compared with the microglial cells found in immediately fixed slices and those scattered in the gray matter. The highest IB_4-immunoreactivity (concerning both staining intensity and cell numbers) was typically found in the dorsal part of the corticospinal tract, in a relatively well defined area where the majority of microglial cells displayed a large, round cell soma and large phagocytic vacuoles representative of the "activated" microglial state (Fig. 7f). Although this could be described as the predominant microglial distribution pattern, numerous alterations concerning the intensity and the distribution of IB_4-immunoreactivity were observed. The number of microglial cells increased in sections derived from the superficial layers of the cultures, while it decreased in sections derived from deeper layers.

Quantitatively, the number of microglial cells reached its peak between 3-6 div and declined after longer culture periods (9-12 div) (Fig. 7g, h; Table 1).

3.1.2.5. Nestin-immunoreactivity persists *in vitro* and is found in cells lining the central canal (Fig. 8 / p. 40-41; Table 1/ p. 42-43)

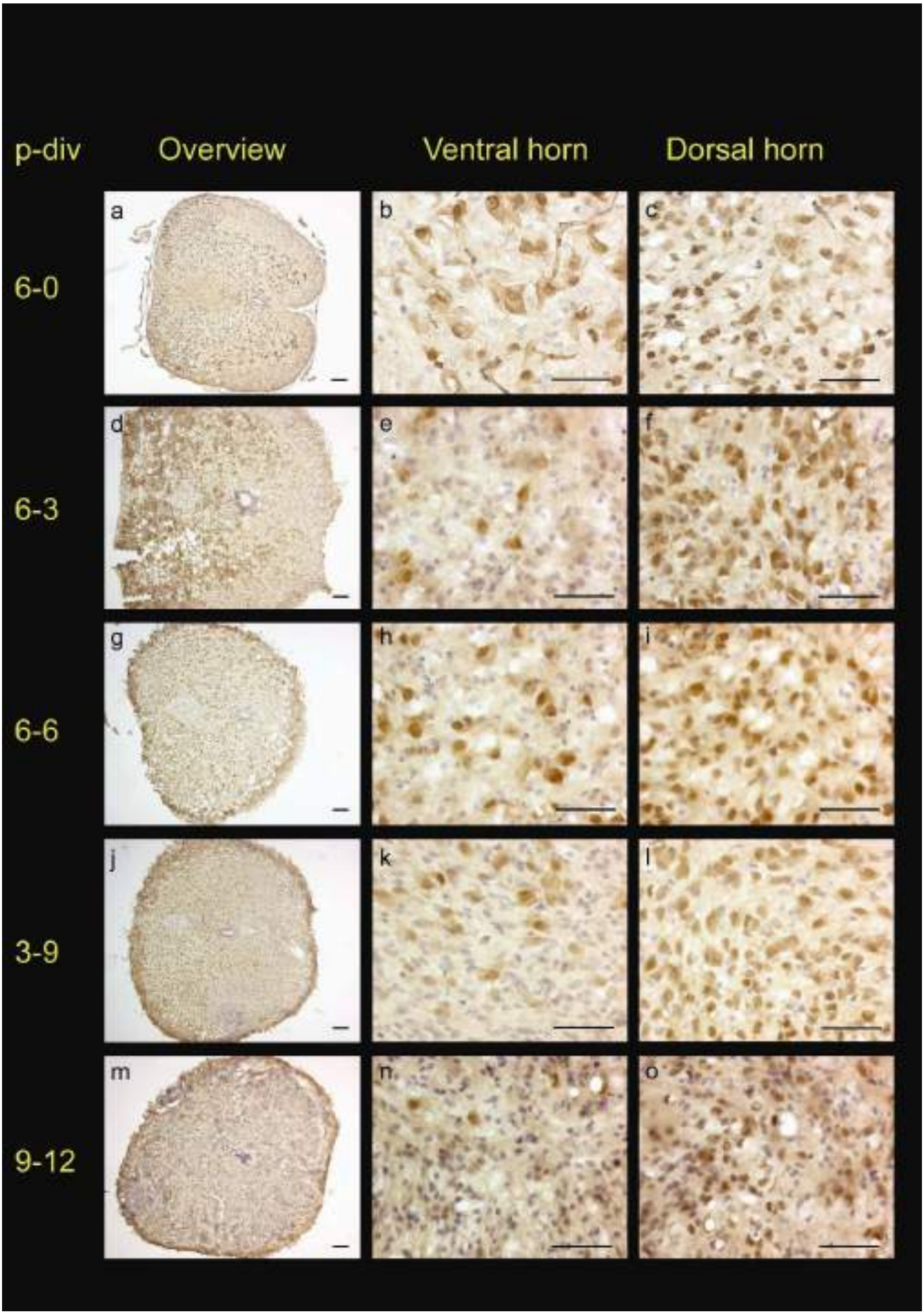
After 3 div an upregulation of nestin-immunoreactivity occurred. Numerous strongly immunoreactive processes extended from the pial surface and spread across the entire spinal cord white matter, even entering the gray matter. The increase was more prominent in SCSC derived from older animals (p9 or p12; Fig. 8m, n), since these demonstrated only weak nestin-immunoreactivity in acutely fixed slices (Table 1). The gray matter contained a relatively low number of $nestin^+$ structures that resembled protoplasmic astrocytes.

Nestin expression in ependymal cells remained elevated, especially in p0 to p6 animals, and a plexus of nestin$^+$ fibers extending towards the surrounding gray matter and being more prominent in the dorsal aspect of the central canal was observed (Fig. 8f, i). This plexus was more intensely labeled in the slices derived from younger animals (p0 to p6). In slices from older animals (p9 or p12) the increase of nestin immunoreactivity prevailed in the white matter (Fig. 8n, q), and only weak nestin-immunoreactivity was found around the central canal (Fig. 8o, r). There was an overall trend for slices cultured for shorter periods (3-6 div) to display more cytoplasmic labeling, whereas longer cultured slices revealed a more diffuse fibre labeling, especially in the ependymal cells.

In summary, in slices derived from p0, p3 or p6 animals, nestin-immunoreactivity remained high or was even upregulated both in the white matter and in the gray matter around the spinal canal (Fig. 8d-i). In slices derived from p9 or p12 animals there was a significant, early, long lasting upregulation of nestin-immunoreactivity in the white matter, while it was less intense around the central canal (Fig. 8m-r). With prolonged culture time the nestin-immunoreactivity was not significantly reduced.

Figure 3: ***NeuN-immunoreactivity in SCSCs***. In SCSCs fixed immediately after explantation (0 div), NeuN$^+$ neurons are distributed across the entire spinal cord gray matter (a). Larger motoneurons are situated in the ventral horn and display strong NeuN-immunoreactivity (b). Smaller, round neurons in the dorsal horn are also NeuN$^+$ (c). After 3 div, the larger motoneurons of the ventral horn are no longer stained (d, e). In contrast, other ventral horn neuronal populations (e, h) and dorsal horn neurons (f, i) are well preserved after 3 div (d-f) and 6 div (g-i). After longer culture periods neurons remain well preserved both in cultures derived from younger animals (j-l) and from older animals (m-o), with the former displaying a better quality of tissue preservation and larger numbers of NeuN$^+$ neurons.

"p-div" refers to the age of the animal at the age of explantation (p) and the number of days *in vitro* (div). Scale bars in all figures: 100 µm.


p-div
Overview
Ventral horn
Dorsal horn
6-0
6-3
6-6
3-9
9-12
a
b
c
d
e
f
g
h
i
j
k
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n
o

Figure 3

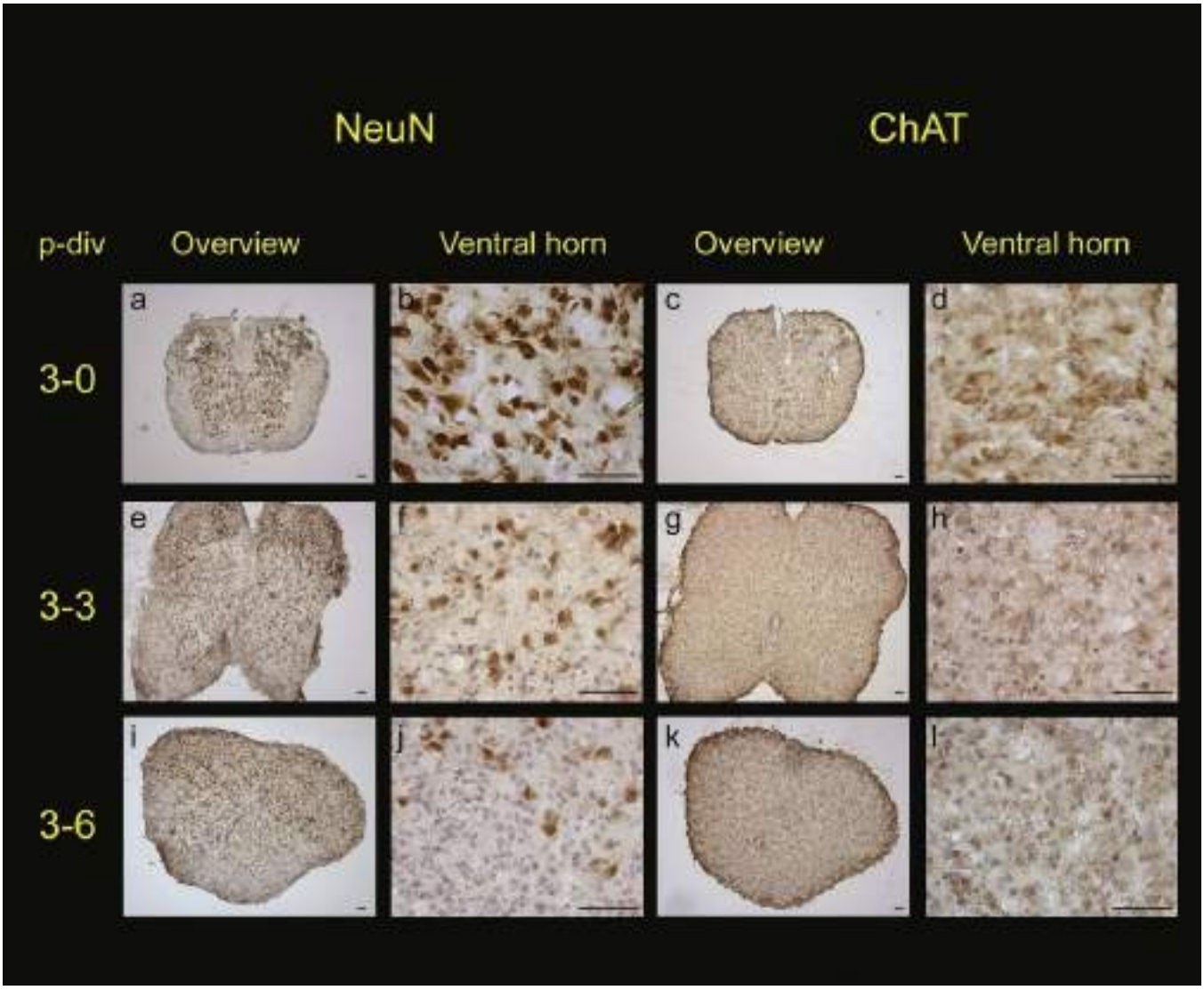

NeuN
ChAT
p-div
Overview
Ventral horn
Overview
Ventral horn
3-0
3-3
3-6
a
b
c
d
e
f
g
h
i
j
k
l

Figure 4

Figure 4: ***ChAT-immunoreactivity in SCSCs***. In acutely fixed SCSCs (0 div) the $NeuN^+$ motoneurons in the ventral horn (a,b) display strong ChAT-immunoreactivity (c,d).After 3 div the number of the $ChAT^+$ motoneurons is reduced, as is the intensity of the remaining ChAT-immunoreactivity (e-h). After 6 div ChAT-immunoreactivity in the ventral horn is barely detectable (i-l), demonstrating the early prominent degeneration of the $ChAT^+$ motoneurons.
"p-div" refers to the age of the animal at the age of explantation (p) and the number of days *in vitro* (div). Scale bars in all figures: 100 µm.

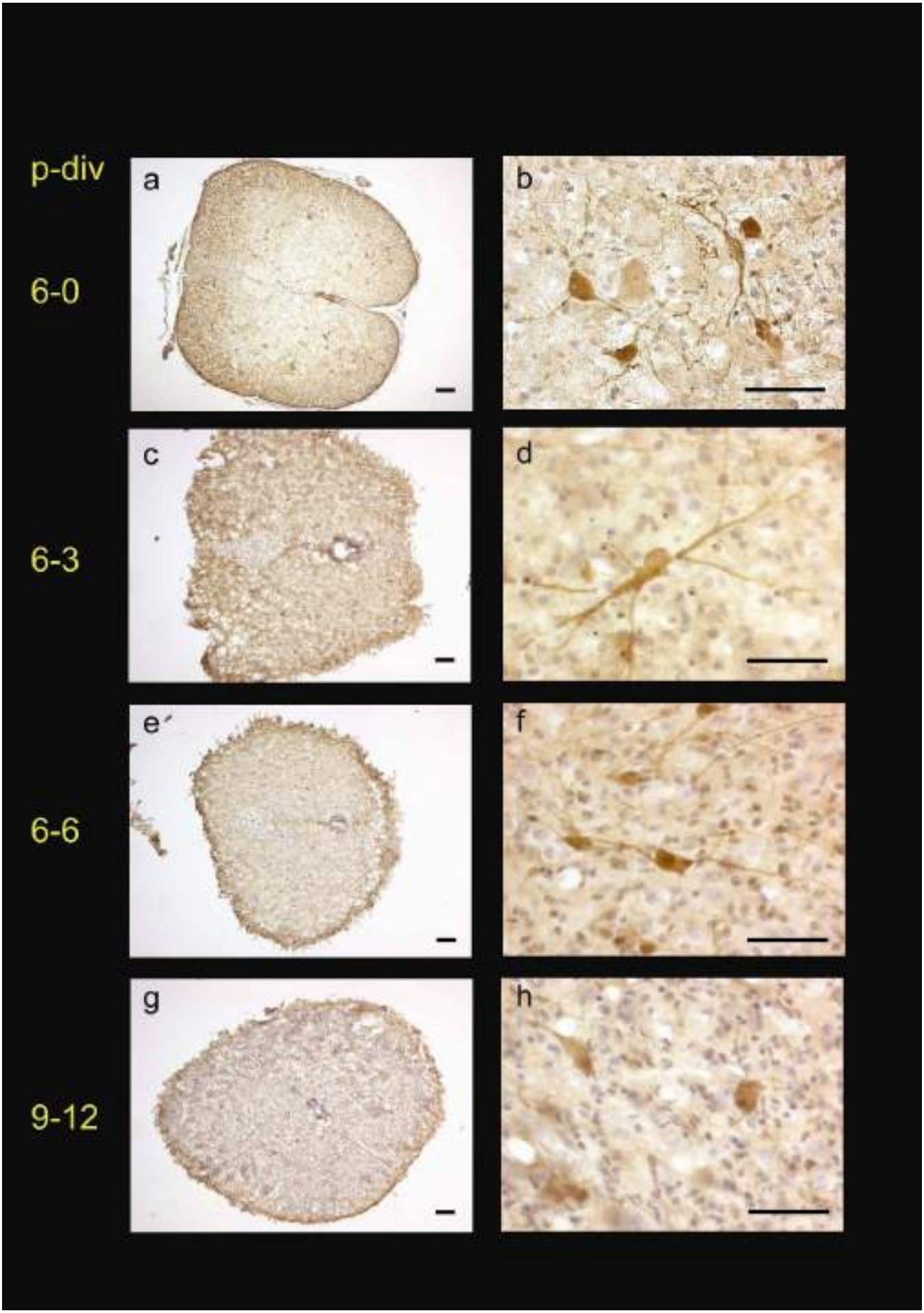


Figure 5

Figure 5: ***Calbindin (CB)-immunoreactivity in SCSCs***. CB-immunoreactivity is found both in the gray and white matter of the spinal cord slices fixed immediately after explantation, with the superficial dorsal horn demonstrating strong CB-immunoreactivity (a). Large CB^+ neurons are found in the ventral horn (b). After 3 div (c, d) or 6 div (e, f) the pattern of CB-immunoreactivity remains well preserved in the superficial dorsal horn. Renshaw cells in the ventral horn (d) and other large CB^+ neurons scattered across the gray matter are accompanied by a dense network of immunoreactive axons and dendrites (f). Ependymal cells are also CB^+ (c, e). After longer culture periods CB^+ neurons are well preserved, even in SCSCs derived from older animals (g, h). CB-immunoreactivity around the central canal remains elevated (g).

"p-div" refers to the age of the animal at the age of explantation (p) and the number of days *in vitro* (div). Scale bars in all figures: 100 µm.

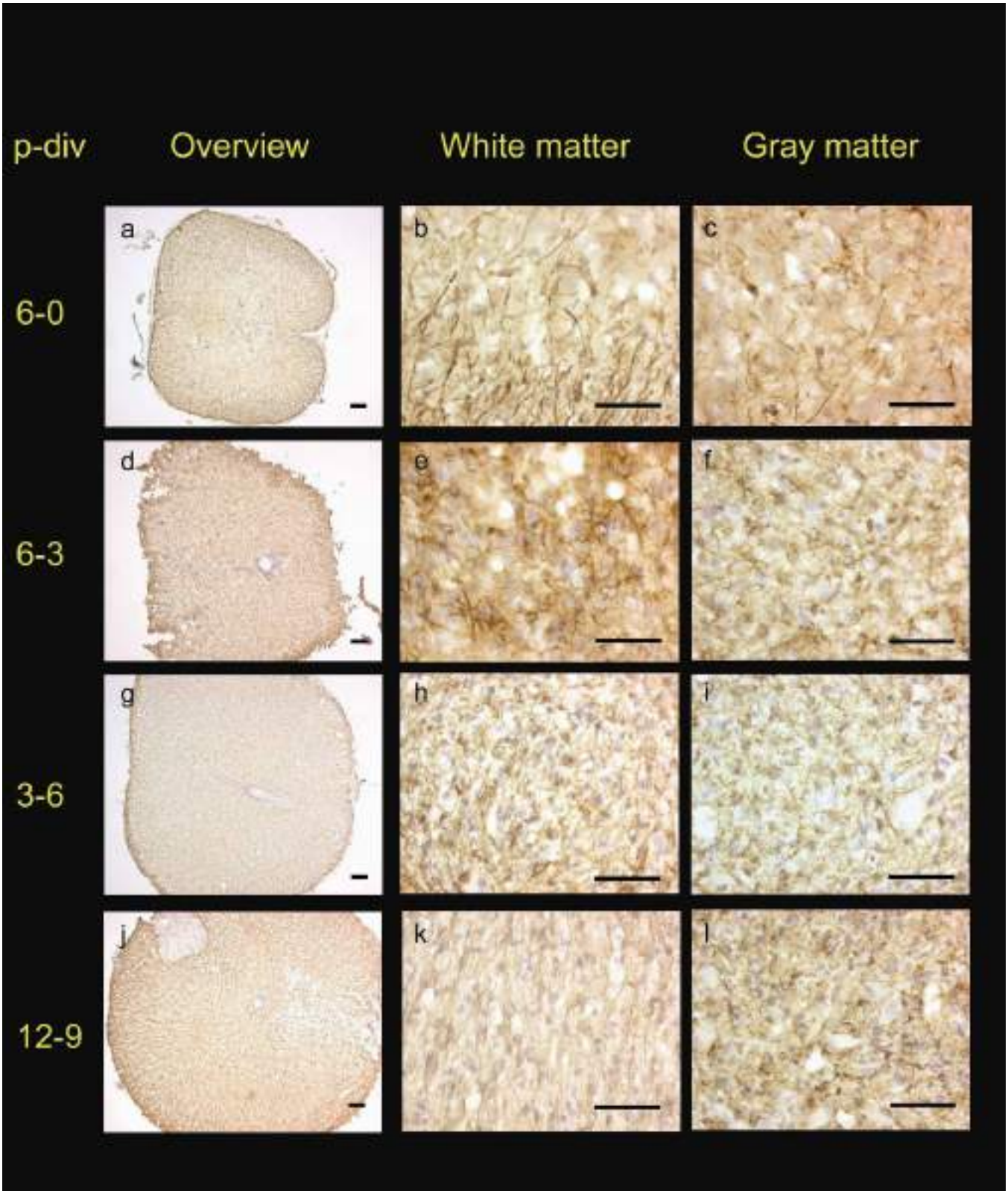

p-div
Overview
White matter
Gray matter
6-0
6-3
3-6
12-9
a
b
c
d
e
f
g
h
i
j
k
l

Figure 6

Figure 6: ***Glial fibrillary acidic protein (GFAP)-immunoreactivity in SCSCs***. In the spinal cord fixed immediately after explantation GFAP-immunoreactivity is almost exclusively restricted to the white matter (a). Astrocytes are oriented radially, spanning the white matter from the pial surface to the interface between gray and white matter (b). Only few astrocytes are present in the gray matter (c). After 3 div (d) GFAP-immunoreactivity remains detectable in the white matter (e), while it is clearly elevated in the gray matter (f). After 6 div (g-i) and 9 div (j-l) the number of white matter astrocytes remains elevated (h, k), and the gray matter further displays numerous strong $GFAP^+$ astrocytes (i, l).
"p-div" refers to the age of the animal at the age of explantation (p) and the number of days *in vitro* (div). Scale bars in all figures: 100 µm.

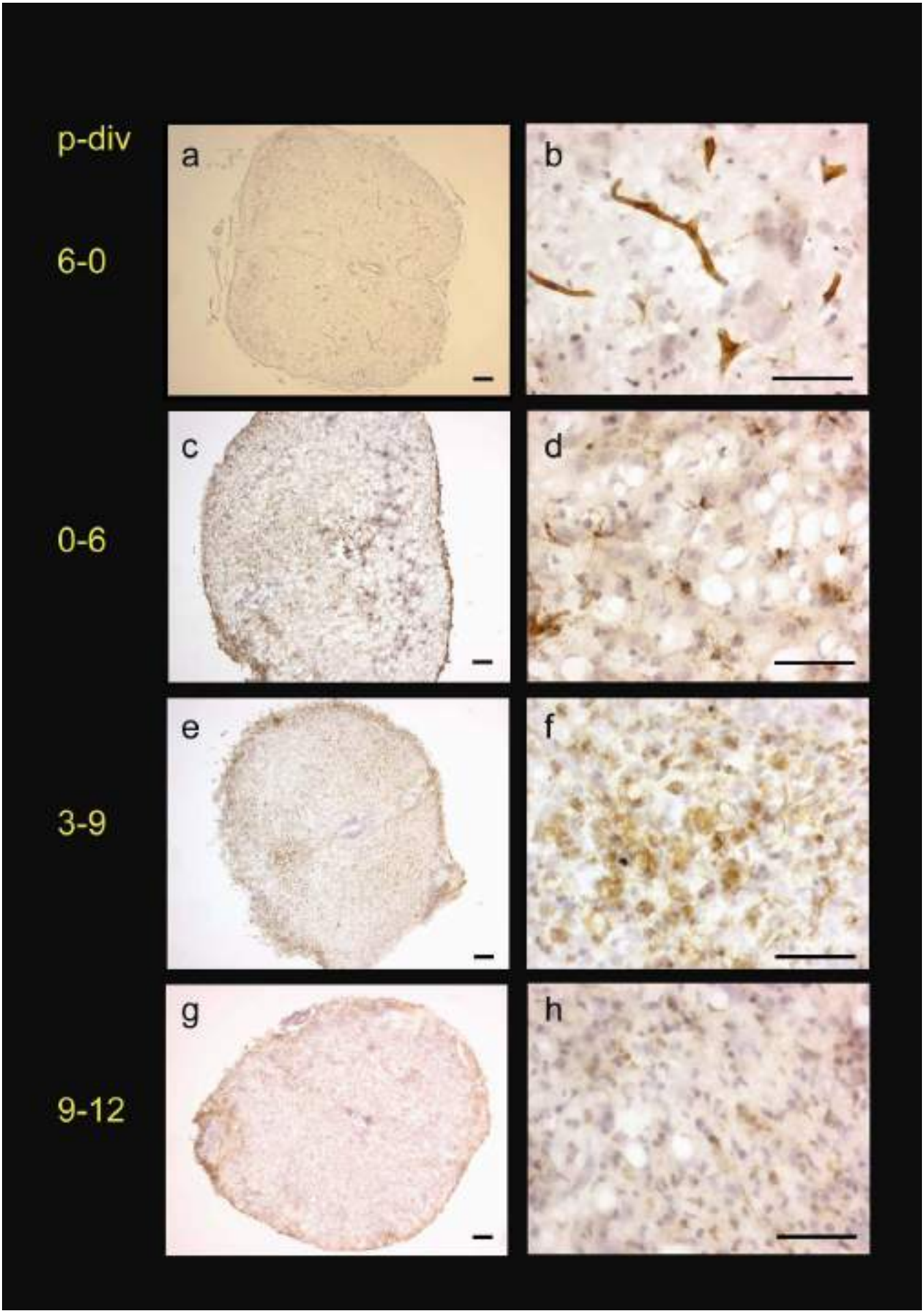
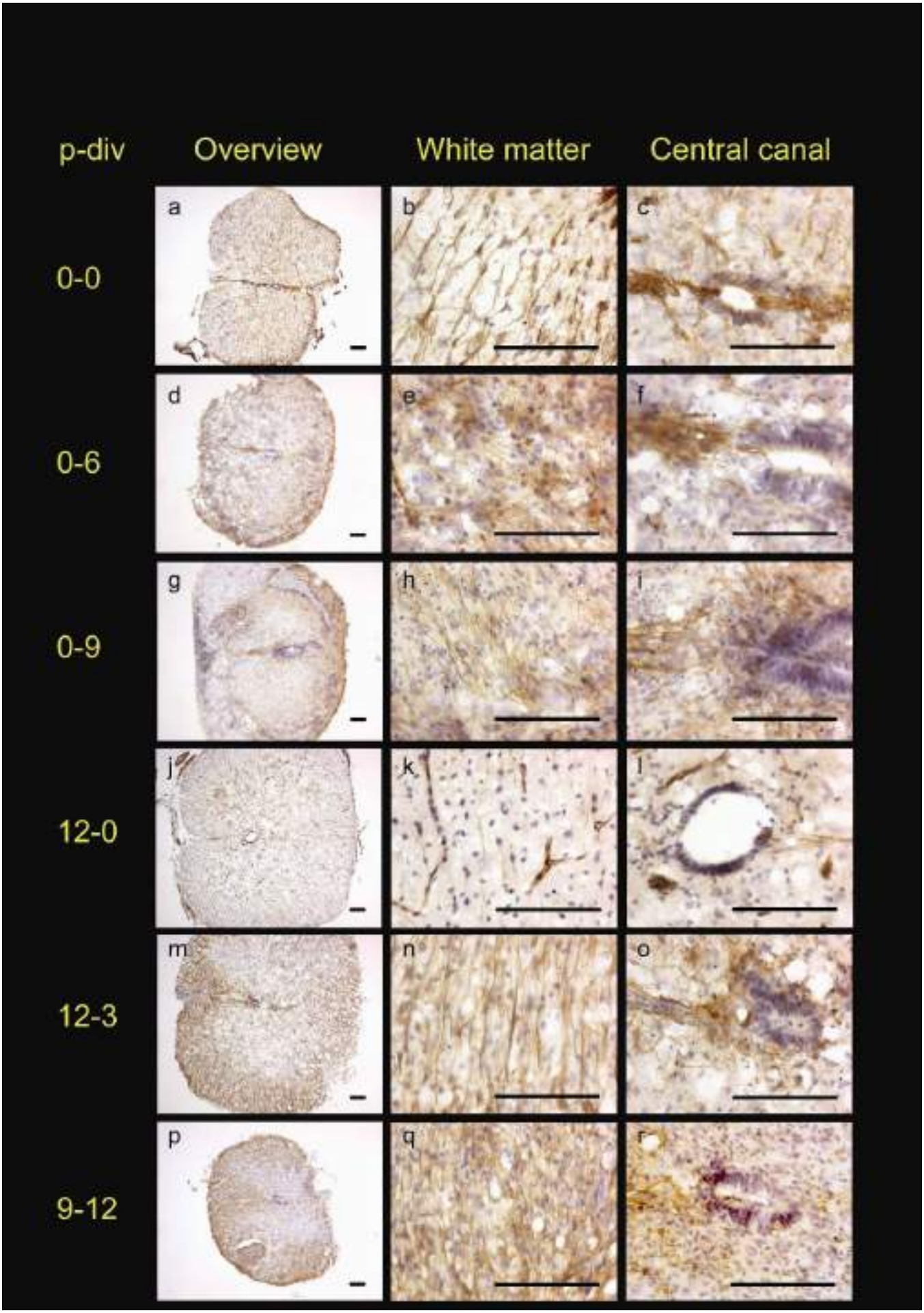


Figure 7

Figure 7: ***Griffonia simplicifolia isolectin B_4 (IB_4)-immunoreactivity in SCSCs***. In the spinal cord investigated immediately after explantation almost no IB_4-immunoreactivity can be found, apart from blood vessels (a, b). In the course of *in vitro* culture, IB_4^+ microglial cells are found predominately in the corticospinal tract and in the white matter (c, e). Some ramified, "resting" microglial cells are scattered across the white and gray matter (d), while the majority of microglial cells demonstrate the round or amoeboid shape typical of "activated" microglia (d, f). After longer culture periods, fewer IB_4^+ cells can be found (g, h). "p-div" refers to the age of the animal at the age of explantation (p) and the number of days *in vitro* (div). Scale bars in all figures: 100 µm.


p-div
Overview
White matter
Central canal
0-0
0-6
0-9
12-0
12-3
9-12
a
b
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d
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f
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Figure 8

Figure 8: ***Nestin-immunoreactivity in SCSCs***. In the spinal cord derived from younger animals (p0 or p3) and fixed immediately after explantation nestin-immunoreactivity is prominent (a), with radially oriented nestin$^+$ cells in the white matter (b). Ependymal cells are nestin$^+$ and there is a bundle of nestin$^+$ cells and fibers spreading towards the ventral and dorsal aspect of the spinal cord (c). In SCSCs derived from younger animals and cultured for 6 div (d-f) or 9 div (g-i), nestin expression remains elevated in the white matter (e, h) and especially around the central canal (f, i). In acutely fixed spinal cord slices derived from older animals (p6 or p12; j-l) only weak nestin-immunoreactivity can be found in the white matter (k) and around the central canal (l). In SCSCs derived from older animals (p6 or p12) there is a rapid, robust and long lasting increase in nestin expression, predominately in the white matter (n, q) and less around the central canal (o, r).
"p-div" refers to the age of the animal at the age of explantation (p) and the number of days *in vitro* (div). Scale bars in all figures: 100 µm.

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Table 1: A cell count of the various cell populations.

P: age in days

div: days *in vitro*

v.h.: ventral horn

d.h.: dorsal horn

w.m.: white matter

g.m.: gray matter

NeuN		0 div	3 div	6 div	9 div
P0	v.h.	95±5.7	89.5±4.63	91.25±2.02	51±5.2**
	d.h.	319.5±6.41	306±7.76	283.5±21.4	255.7±3.53
P6	v.h.	69.67±4.06	62±1	48±2.65	25.67±2.91**
	d.h.	252.3±6.39	260.5±1.5	231.3±12.67	250.8±20.72
P9	v.h.	94±9.24	61.5±1.5	23±2***	12.25±0.48***
	d.h	292.7±3.49	193±7	162.5±18.5**	123.3±20.95***

Calbindin	0 div	3 div	6 div	9 div
P0	73±7.57	63±2	44±2.78	39.25±2.66*
P6	75±5.03	79±7	44.67±4.41	31±7.43***
P9	89±6.25	42±1**	48±3.79**	20±2.74***

GFAP		0 div	3 div	6 div	9 div
P0	w.m.	33.67±0.88	30±1	30±3.79	31±1.16
	g.m.	5.33±0.88	31±2.31*	29±2.08*	31.33±2.19*
P6	w.m.	47.6±2.87	43.75±2.27	41.75±1.55	39±0.71
	g.m.	21.2±1.72	65.63±3.27***	63.67±4.36***	70±5.29***
P9	w.m.	39.33±1.45	33.67±0.88	34.67±2.4	33.67±1.76
	g.m.	23.67±1.33	65±4.62***	77±3.46***	92.33±2.03***

IB_4	0 div	3 div	6 div	9 div
P0	16.33±2.33	183.5±12.5***	80.5±11.65*	56.67±6.49
P6	17±1.53	141.2±17.01***	71.6±4.4	45.4±4.21
P9	24±1.15	72.33±9.94	124.2±6.89***	67.8±8.25

Nestin	0 div	3 div	6 div	9 div
P0	27±1.15	18.8±1.78	15±1.73	10.5±0.64***
P6	12.33±0.88	35.25±3.68***	17±1.58	15.33±2.33
P9	7.5±0.5	27±1**	17±1	13.5±1.5

* = $p<0.05$ ** = $p<0.01$ *** = $p<0.001$

3.2. PART II: ESTABLISHING A SLICE CO-CULTURE MODEL OF MOTORCORTEX AND SPINAL CORD

After the characterization of defined neuronal and glial populations in organotypic slice cultures from rat spinal cord described in the first part of our study, we co-cultured rat motorcortex and spinal cord slice cultures and combined this with anterograde axonal labeling. This co-culture system was then used to investigate effects of pia and white matter removal and of exogenously administered NT-3 on neuronal survival and axonal sprouting.

Axonal labeling following Mini-Ruby application on the layer V of the motorcortex was visible in fluorescence microscopy during the period of *in vitro*-culture, and following fixation and preparation for light microscopy after 4 div. Neurons labeled with MiniRuby were clearly seen in the motorcortex. The anterograde labeling was characterized by discretely labeled somata, dendritic processes and networks, and axons demonstrating terminal arborizations, varicosities, bouton-like specializations and growth cones. Labeling was overall satisfactory after 4 div following Mini-Ruby application, and revealed differences between the various experimental groups.

3.2.1. Almost no sprouting axons are found in the control group

After 4 div the number of surviving neurons in the motorcortex of the control group was small. A very limited number of pyramidal cells and axons labeled with MiniRuby was visualized (Fig. 9a, b / p. 46-47). The cytoarchitecture was poorly preserved especially in the motorcortex, while the spinal cord tissue seemed to be better preserved.

In the spinal cord ventral horns, cells of a size corresponding with small and medium size motoneurons were visible, whereas large motoneurons seemed absent. Large quantities of dorsal horn interneurons persisted. We observed very few labelled axons, originating from the motorcortex, in the area surrounding the corticospinal interface of the “whole slice” subgroup. These sprouting axons were rare, short and limited to the motorcortex. No sprouting axons crossing the corticospinal interface or entering the spinal cord tissue were observed (Fig. 10a, b / p. 48-49).

In the “half-slice” subgroup, where spinal cord gray matter was directly opposed to the cortical cultures and culture was performed in control medium, there was no significant improvement in the amount of axonal sprouting.

3.2.2. Effect of exogenous NT-3 application: Sprouting axons are observed in the NT-3 group

In contrast to the control group, after 4 div in the NT-3 group, an improved survival of cortical neurons was seen, correlating with an improved cortical cytoarchitecture. Numerous MiniRuby-labeled neurons were found in all layers of the motorcortex, demonstrating a healthy morphology. They were accompanied by a fiber network consisting of Mini-Ruby labeled dendrites and axons (Fig. 11a, b / p. 50-51). As in co-cultures treated with control medium, the preservation of spinal cord morphology was generally good. Anterograde labeling of neurons in the motorcortex resulted in visualization of sprouting axons that were longer than in the control group.

In the area of the corticospinal interface many Mini-Ruby-labeled corticospinal sprouting axons were observed. In the "whole slice" subgroup the axons grew towards the corticospinal interface, but changed their course, eventually demonstrating a parallel course to the interface (Fig. 12a, b / p. 52-53). They were unable to cross the corticospinal interface, consisting of pia and white matter, and did not enter the spinal cord.

In the "half slice" subgroup treated with NT-3 the findings were strikingly different: Sprouting axons clearly crossed the corticospinal interface and entered the spinal cord tissue. Sprouting axons with well formed growth cones were observed, demonstrating a maximal length of 500 µm (Fig. 13a, b / p. 54-55).

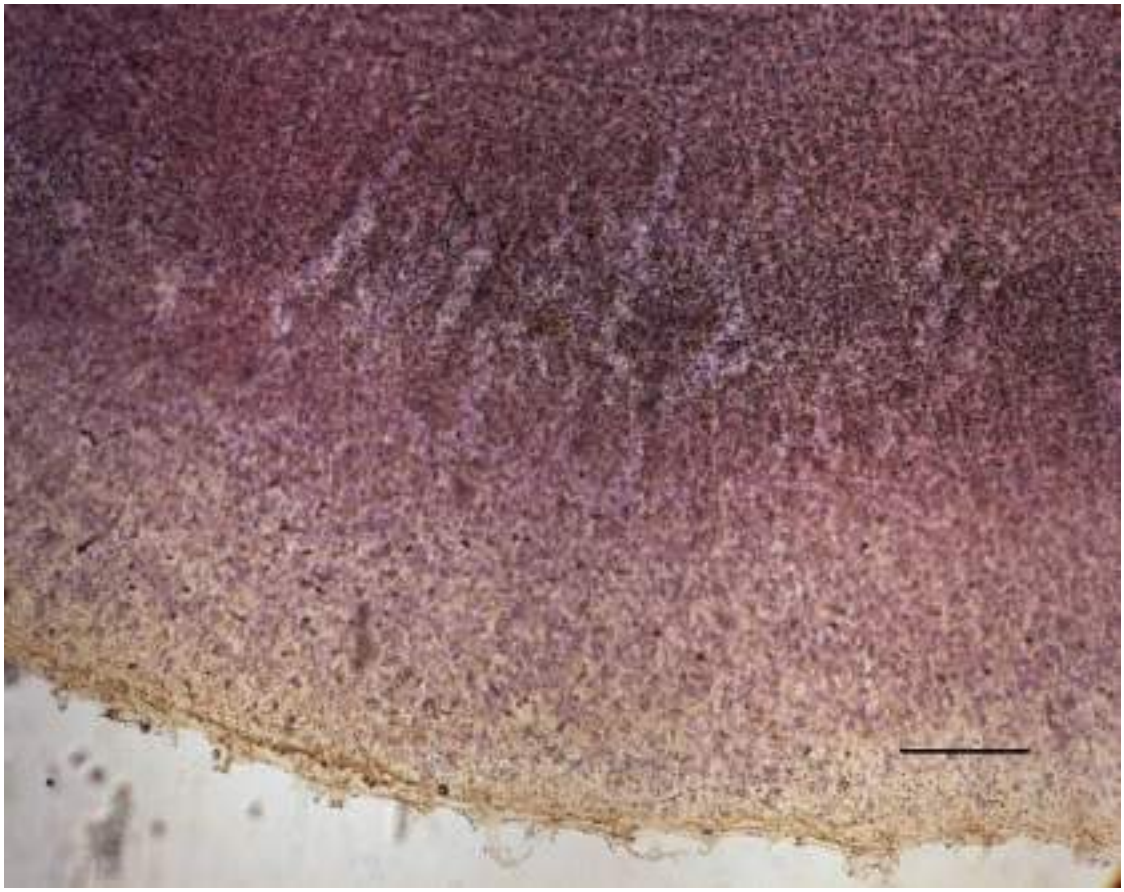


Figure 9a

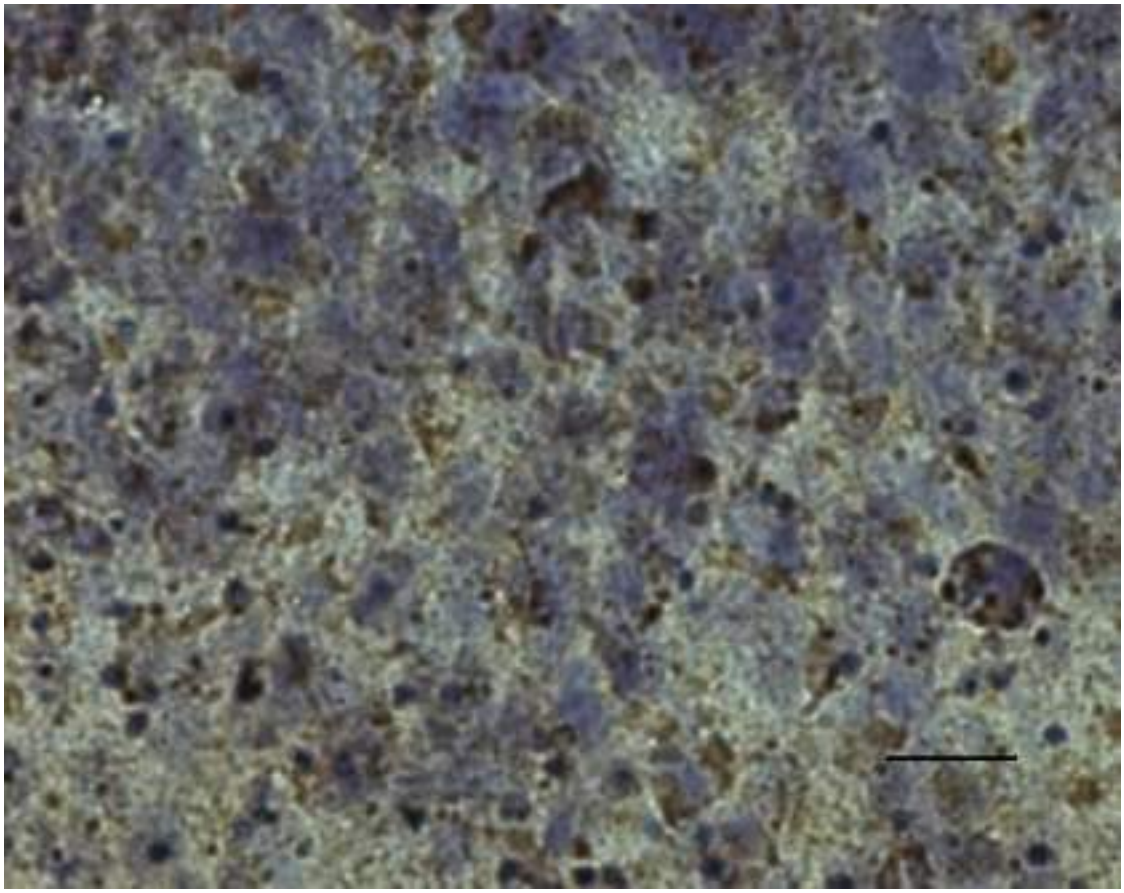


Figure 9b

Figure 9a, b: In the control group, the number of surviving motorcortex neurons is low. Only few Mini-Rubi labeled neurons and axons can be seen after 4 div, and the cortical cytoarchitecture in the control group is poorly preserved.
(Scale bar in Fig. 9a:400 µm, in Fig. 9b: 50 µm)

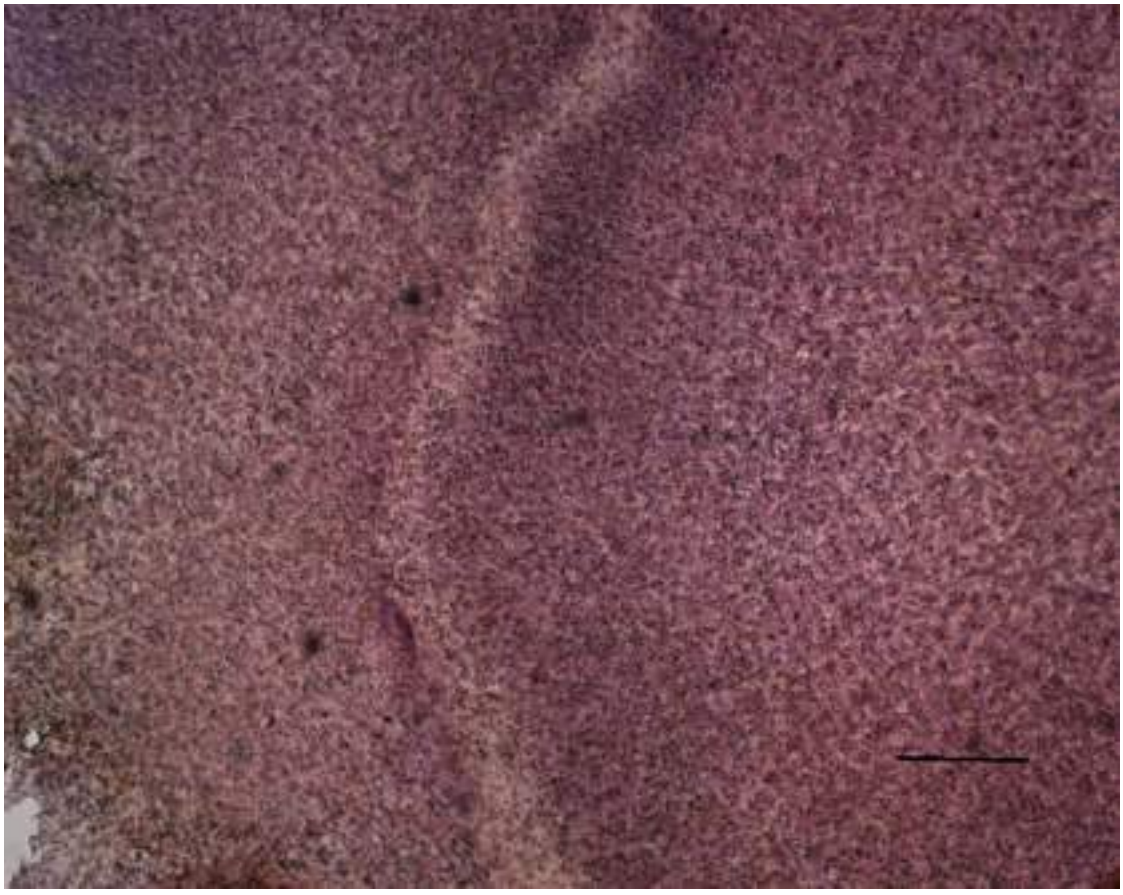


Figure 10a

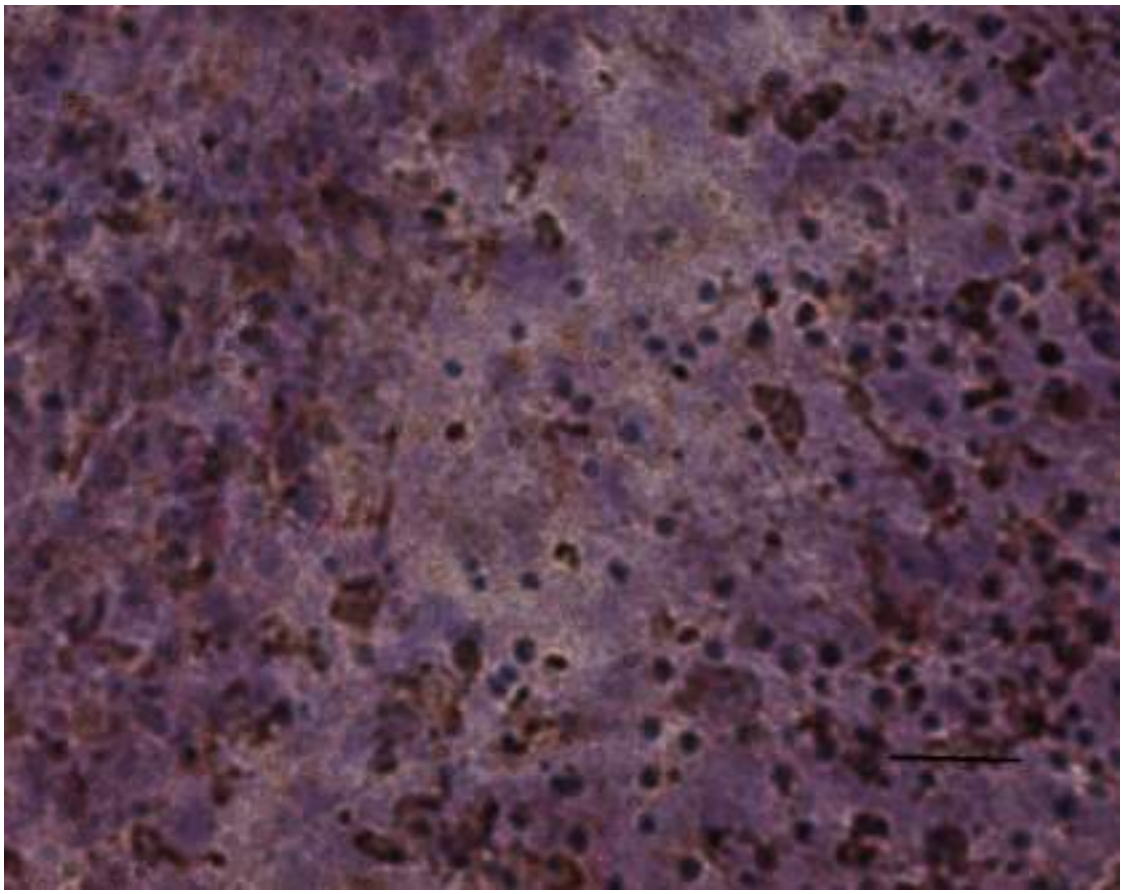


Figure 10b

Figure 10a, b: In the group treated with control medium, very few sprouting axons are observed in the “corticospinal interface” region.
(Scale bar in Fig. 10a:200 µm, in Fig. 10b: 50 µm)

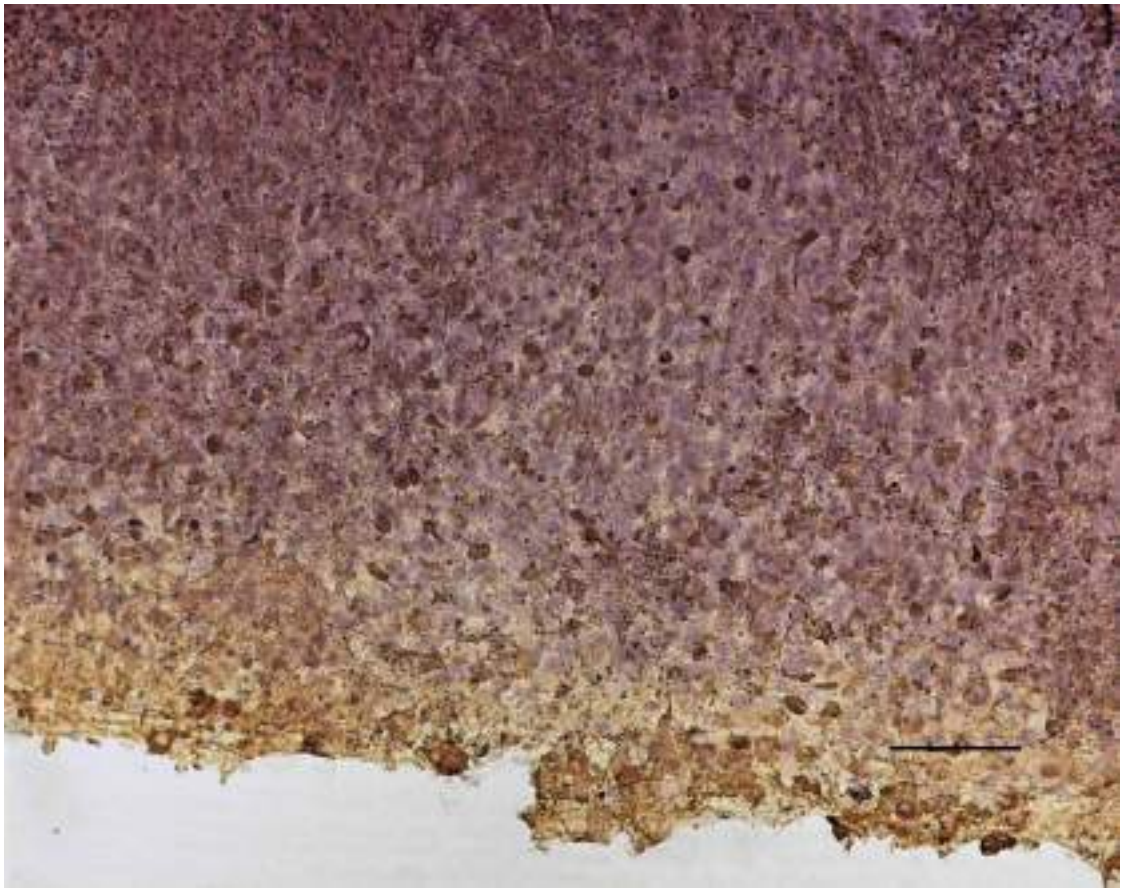


Figure 11a

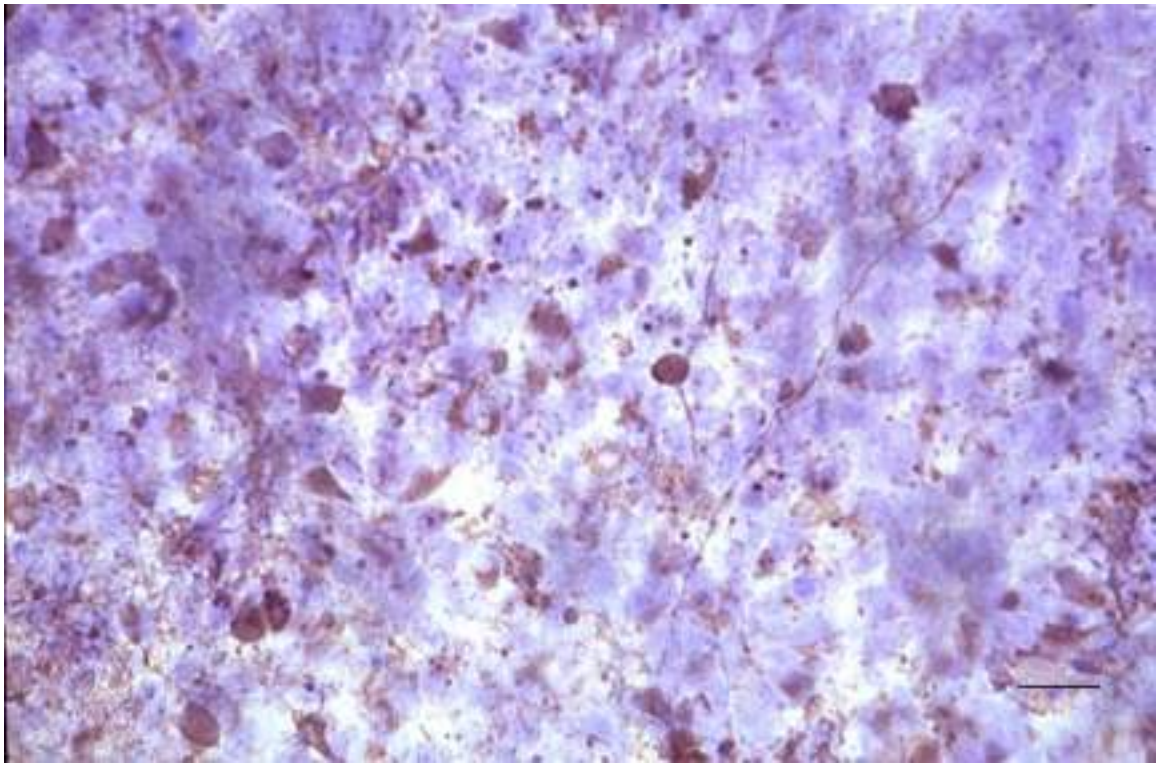


Figure 11b

Figure 11a, b: Compared to the control group (Fig. 9) motorcortex neurons and the cortical cytoarchitecture in the NT-3 group are better preserved after 4 days *in vitro*. Anterograde labeling of cortical neurons with the dextran amine MiniRuby results in visualization of neurons in all layers of the motorcortex (11a). The neurons demonstrate a healthy morphology and are accompanied by a well defined fiber network consisting of their dendrites and axons (11b).
(Scale bar in Fig. 11a:400 µm, in Fig. 11b: 40 µm)

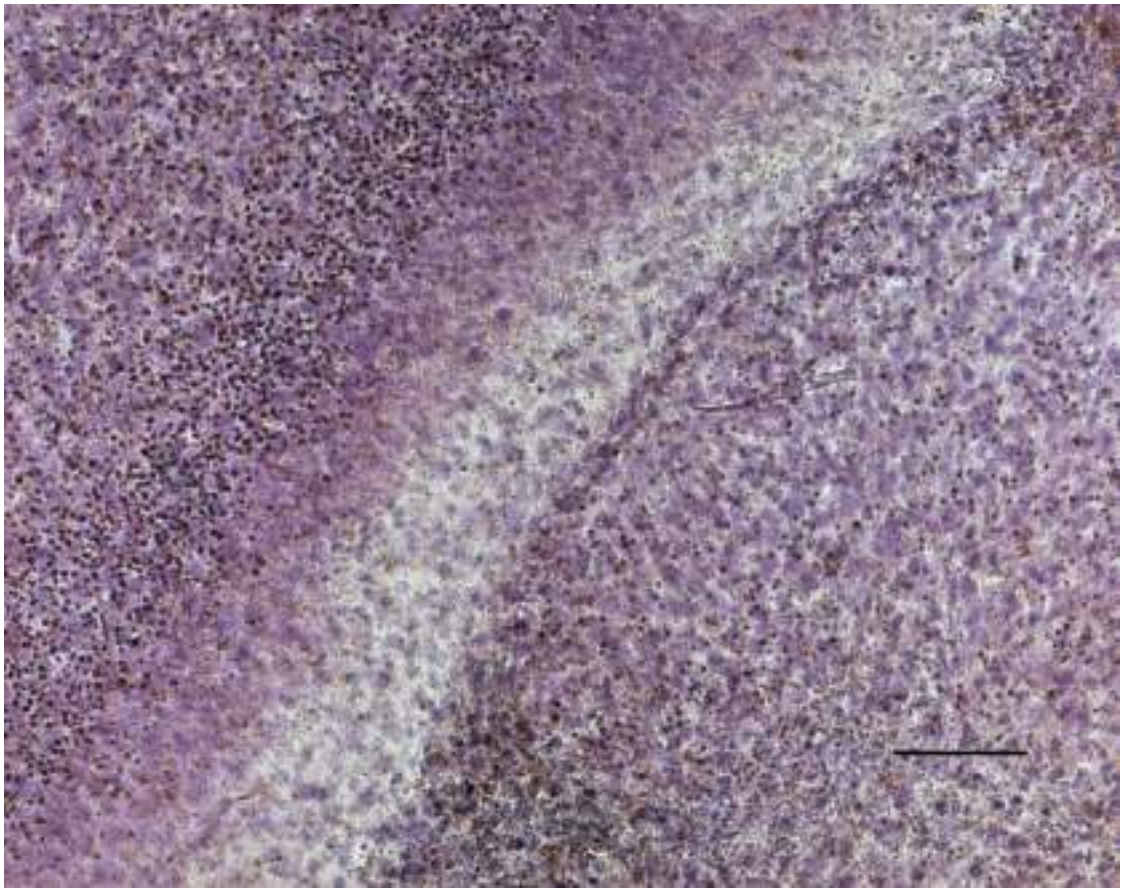


Figure 12a

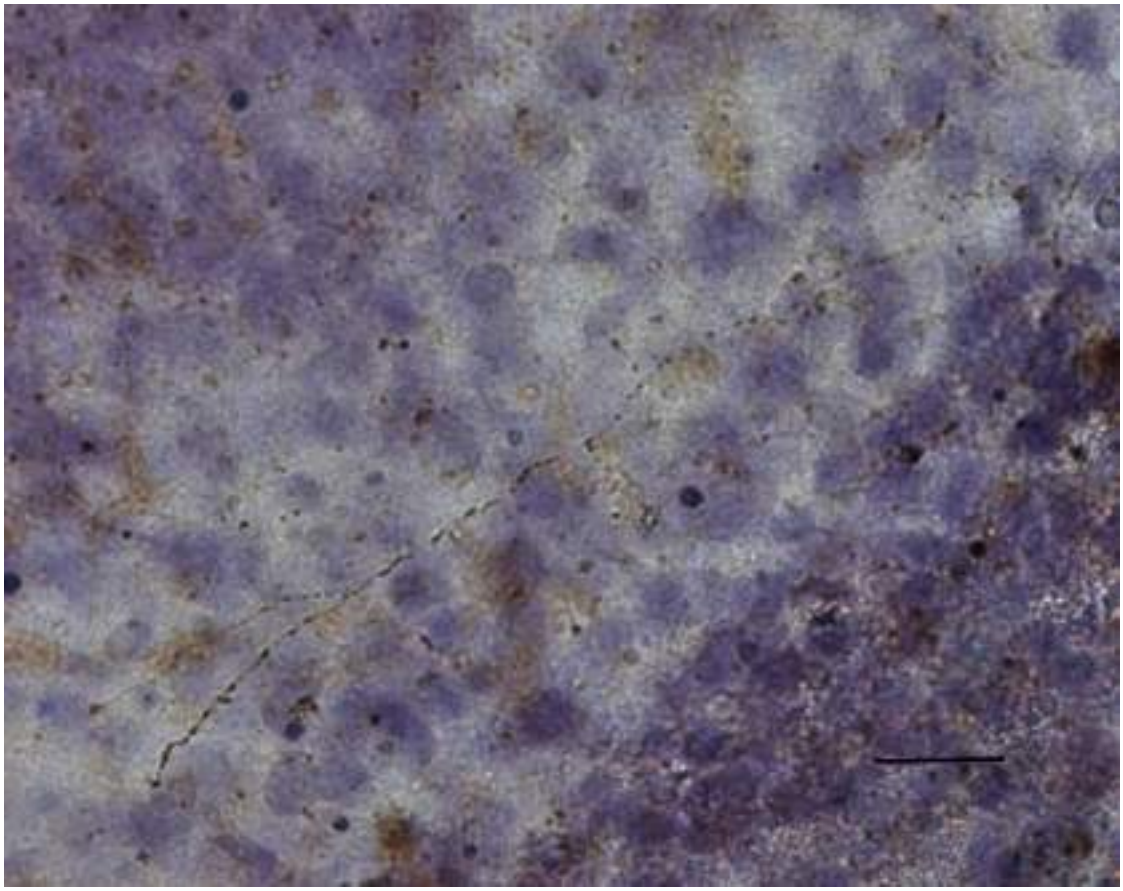


Figure 12b

Figure 12a, b: After 4 div, in the “whole slice” subgroup treated with NT-3, an axon grows towards the corticospinal interface, finally demonstrating a course parallel to it, but it does not enter the white matter of the spinal cord itself.
(Scale bar in Fig. 12a:200 µm, in Fig. 12b: 40 µm)

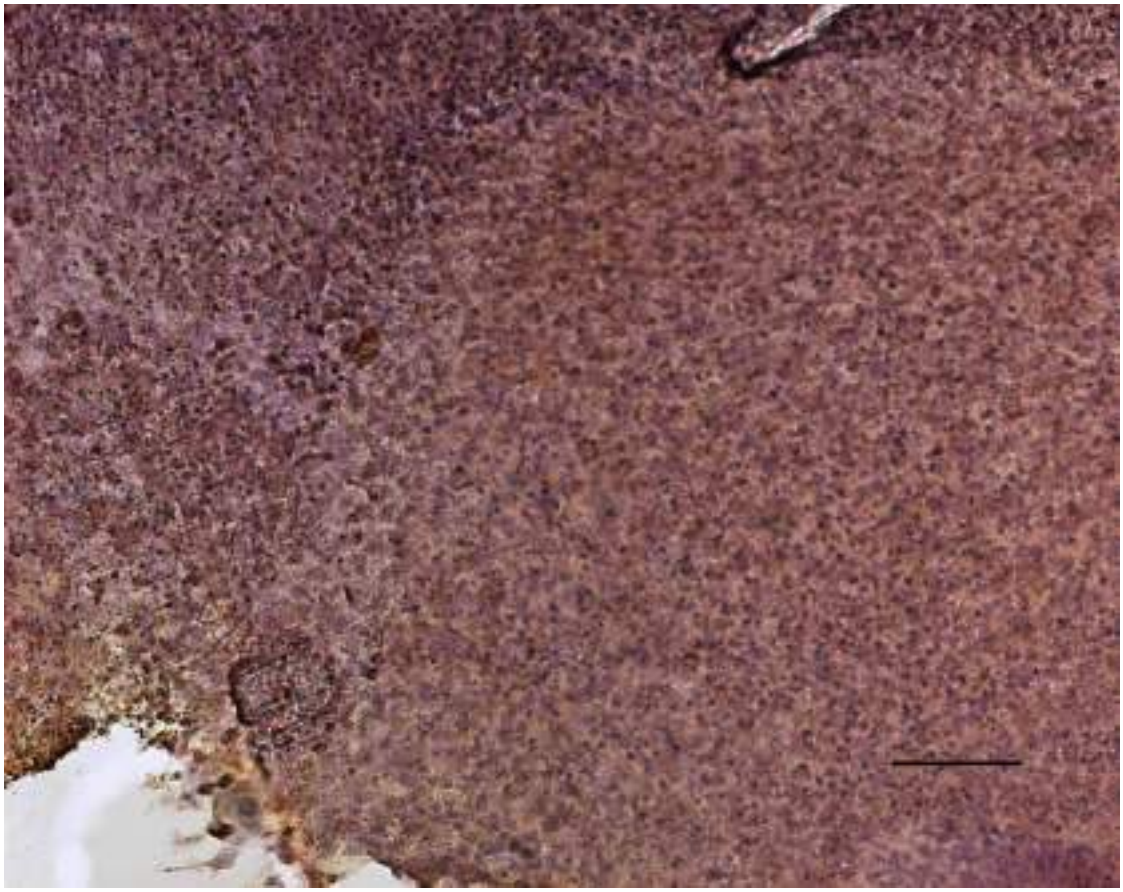


Figure 13a

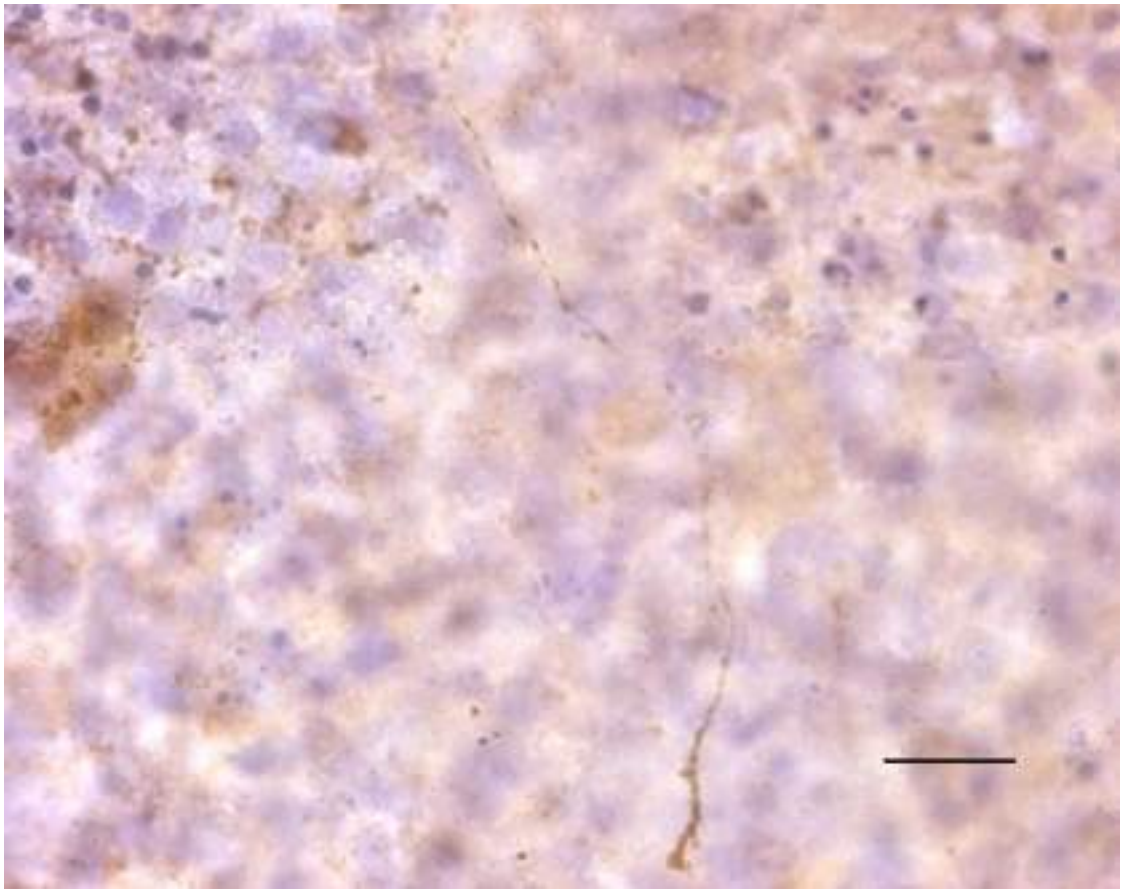


Figure 13b

Figure 13a, b: After 4div in the “half slice” subgroup treated with NT-3, a sprouting axon traverses the corticospinal interface and enters the gray matter of the spinal cord. A sprouting axon with a growth cone and a maximal length of 500 µm is found.
(Scale bar in Fig. 13a:400 µm, in Fig. 13b: 40 µm)

4. DISCUSSION

In the first part of our study we investigated spinal cord slice cultures (SCSCs) derived from rats of different ages in order to examine the suitability of these preparations as an *in vitro* model for degenerative and regenerative reactions after SCI.
In the second part, which is discussed separately, it was our aim to establish a functional *in vitro* model suitable for the investigation of effects of neurotrophic factors on neuronal survival and axonal sprouting in the corticospinal system.

PART I: CHARACTERIZATION OF SPINAL CORD SLICE CULTURES

4.1. ADVANTAGES AND DISADVANTAGES OF OSCs

Slice cultures from different regions of the CNS can be maintained *in vitro* for several weeks. Cells within such preparations preserve the tissue-specific cell connections that exist *in vivo*, as well as local neuronal circuits with the appropriate patterns of innervation. It is well established that neuronal as well as non-neuronal cells are well preserved in OSCs. Neurons in OHSCs are known to preserve morphological and physiological features of the *in vivo* situation, while microglial cells in OHSCs are initially activated following explantation but return to a resting state after at least 6 div (Hailer et al, 1996).
Furthermore, the more complex parameters that complicate studies *in vivo*, such as blood supply and the existence of the blood brain barrier, are excluded in OSCs. The combination of pharmacological accessibility and long-term survival along with preservation of regional differentiation, neuroanatomical organization and cell-to-cell interaction renders organotypic slice cultures a very useful model in the study of neuronal tissue (Bernaudin et al, 1998).
A problem emerging in the case of SCSC is the unavoidable interruption of all afferent and efferent neuronal pathways. “Target dependency” seems to be an important aspect of neuronal survival, especially in immature neuronal tissue. Developmental cell death has been observed in numerous cell groups in the developing vertebrate nervous system, whereas only a few types of neurons apparently do not undergo developmental cell death. Two common characteristics are that cell death occurs during a specific period of development and that -with few exceptions- it is regulated by postsynaptic target tissue. For most types of somatic motoneurons cell death is initiated if neurons do not receive an adequate stimulus from postsynaptic targets during a critical developmental stage (Wetts et al, 1998).
Moreover, it is reasonable to assume that tissue preparation in order to obtain slice cultures equals a severe tissue trauma that triggers the activation of microglial cells and astrocytes.
Taken these facts into consideration, we have investigated the spatiotemporal course of distinct neuronal and glial populations, using well established immunohistochemical markers.

We examined whether the slice culture of postnatal rat spinal cord is an *in vitro* model where defined neuronal and glial populations remain preserved over longer culture periods. Moreover, we studied OSCs derived from animals of different ages and sustained *in vitro* over different time periods, in order to investigate the influence of a) the animal age at the time of explantation, and b) the duration of the *in vitro* period on tissue preservation of spinal cord OSCs.

Our findings on the temporal course of the distinct cell populations of the slice cultures are in agreement with most *in vivo* experiments, as *in vitro* cellular responses correlate well with *in vivo* findings after experimental spinal cord lesions.

4.2. THE FATE OF NEURONAL POPULATIONS IN SCSCs

The number, morphology and distribution of smaller $NeuN^+$ neurons in the gray matter of SCSCs appear to remain well preserved, even after prolonged culture periods. In the following discussion the preservation pattern of CB^+ neurons and larger $ChAT^+$ motoneurons are discussed in detail, since these neuronal subgroups were studied extensively in our experiments.

4.2.1. Preservation of calbindin$^+$ neurons

CB^+ neurons show a capacity for survival over prolonged culture periods, although their numbers decline significantly. The majority of CB^+ neurons in the dorsal horn presumably represent local interneurons (Nazli et al, 2000). Since the superficial dorsal horn is an important relay station for nociceptive input with most nociceptive primary afferents terminating in laminae I and II, it is most likely that these CB^+ interneurons play an important role in nociceptive processing and transmission (Ren et al, 1994).

In the ventral region of lamina VII the medium- to large-sized CB^+ neurons with long processes are suspected to be Renshaw cells. Renshaw cells are an heterogeneous population of interneurons, consisting of two morphological types, multipolar and fusiform. Renshaw cells use GABA and/or glycine as neurotransmitters, receive diverse convergent inputs and project to numerous cell populations (α-motoneurons, γ-motoneurons, other interneurons). One of their main functions is to mediate postsynaptic, recurrent inhibition, since they predominantly establish contact on the dendrites of their target motoneurons in lamina IX. In the rat, all neurons classified as Renshaw cells also are CB^+, and all CB^+ cells located in the ventral-most region of lamina VII express the characteristic gephyrin labeling and morphology of Renshaw cells (Carr et al, 1998).

4.2.1.1. The neuroprotective effect of calbindin

Calbindin (CB) is a member of the EF-hand family of calcium binding proteins. It has been proposed that, because of their calcium buffering capacity, these proteins may protect neurons against potentially harmful increases in intracellular calcium ions (D'Orlando et al, 2001; Iacopino et al, 1992; McMahon et al, 1998; Rintoul et al, 2001). Abnormal high and sustained increases in the intracellular calcium ion concentration ($[Ca^{2+}]i$) may activate catabolic enzymes, including protein kinase C, calpains, phospholipase A_2, nitric oxide synthase and endonucleases, the consequence of which is necrotic or apoptotic cell death. Even more important, Ca^{++} influx induces substantial mitochondrial Ca^{++} loading, leading to a collapse of mitochondrial membrane potential during early necrotic phase. As the increase in $[Ca^{2+}]i$ plays a key role in excitotoxic cell death, neurons that express calbindin or the other calcium binding proteins may be more resistant than those that do not.

Especially Renshaw cells contain high levels of CB suggesting the presence of a specialized calcium regulatory mechanism. This is of particular importance, as Renshaw cells are high-frequency inhibitive interneurons that need to efficiently control intracellular calcium fluxes arising from high frequency bursts of action potentials.

It has been shown, that after unilateral spinal nerve transection in p3 rats and despite loss of 80% of their target motoneurons there was no significant loss of cells among interneurons and especially in the Renshaw cells region after 3 and 18d. This resistance of interneurons to cell death could -at least in part- be explained by their complex connectivity, which renders their survival in culture less target dependant, and by the protective potential of calcium binding proteins (Lim et al, 2000).

Our finding, that CB^+ Renshaw-cells were well preserved over longer culture periods, even after the larger $ChAT^+$ motoneurons had disappeared, is in accordance with these *in vivo* findings.

4.2.2. Early loss of large motoneurons in the ventral horn

In contrast to this relatively high degree of preservation of smaller $NeuN^+$ or CB^+ neurons, SCSCs demonstrate a significant and early loss of large motoneurons in the ventral horn. Already after 3 div these $NeuN^+$ and $ChAT^+$ neurons display signs of degeneration, accompanied by a large reduction of ChAT-immunoreactivity in SCSCs, although other $NeuN^+$ and CB^+ ventral horn neurons are still present. The role of different factors influencing neuronal survival, such as afferent and efferent connections and neurotrophins, has to be discussed:

4.2.2.1. The role of afferent connections in neuronal survival

It has been argued that targets and afferents interact in the control of neuronal survival (Oliveira et al, 2002). Total deafferentation of a neuronal population is difficult to achieve

and to study in the developing nervous system, because of the complexity of afferent innervation and the reinnervation induced by the increased plasticity. It seems that afferent input is important for neuronal survival during development, since deafferentation increases neuronal death by 20-30%, while increasing input diminishes neuronal death.

Neuronal survival is maintained by any input, such as reinnervation by inappropriate fibers; but for optimal survival, morphological maturation and the acquisition of normal physiology, the correct input is required. Afferents maintain their target neurons via a combination of electrical activity and delivery of trophic agents, thereby influencing protein synthesis, mitochondrial function and suppressing apoptosis. Evidence from animal and *in vitro* experiments indicates that afferents play an extremely important role in the survival of developing neurons in the immature vertebrate nervous system (Sherrard et al, 1998).

4.2.2.2. The role of targets in neuronal survival

Neuron-target interactions are easier to interrupt experimentally and have been extensively studied.

Target deprivation of immature motoneurons results in massive neuronal death, especially in the first days of postnatal life. However, this susceptibility declines during the first two weeks of postnatal development and developing motoneurons begin to express a regenerative capacity up p14. Transplantation of a peripheral nerve graft was unable to rescue motoneurons from degeneration if spinal root avulsion was performed in animals younger than p14. However, this procedure enhanced motoneuron survival when root avulsion was performed at p14 or later. The percentage of regenerated motoneurons increased from p21 to p28 and was similar to that of adult animals. Therefore, the regenerative capacity of rat spinal motoneurons seems to begin around p14 (Chan et al, 2002).

Unilateral spinal nerve transection in p3 rats resulted in a marked reduction in the number of ventral horn motoneurons compared to the unlesioned side already after 3 days (Lim et al, 2000).

It is believed that axonal lesion close to the cell body is followed by a more rapid onset and greater neuronal degeneration than in the case of a distal lesion. Non-target support may therefore be important in motoneuronal survival. Such non-target support might mainly come from the peripheral nerve components such as Schwann cells. Since root avulsion results in the complete deprivation of target and non-target supports, it leads to severe motoneuron loss both in early postnatal and adult animals (Wu et al, 1995; Yuan et al, 2000).

Regarding this target and non target support, our SCSC model bears close similarities to the root avulsion model. Since slice culture preparation equals an unavoidable proximal axotomy, the observed early loss of larger ventral horn motoneurons is an expected finding.

4.2.2.3. The role of neurotrophins in motoneuronal survival and developmental cell death

It is well documented that neurotrophic factors from various sources (including skeletal muscles as target tissues, afferent neuronal connections, glial cells, etc.) function together in regulating survival and functional integrity of motoneurons during both embryonic and postnatal development. Many neurotrophic factors have been identified and characterized on a molecular level. From the neurotrophin family, at least three members, BDNF, NT-3 and NT-4, but not NGF, support motoneuron survival. Motoneurons are also supported by other families of neurotrophic molecules, such as the Ciliary Neurotrophic Factor/Leukemia Neurotrophic Factor (CNTF/LIF) family, the Hepatocyte growth Factor/Scatter Factor (HGF/SF), the Insulin-like Growth Factors (IGFs), GDNF, and related factors. Neurotrophic factors may potentiate each other in supporting motoneuron survival. The high number of neurotrophic factors that act on motoneurons indicates that regulation of motoneuron survival and function is a highly complex process. In addition, some of these molecules are only expressed postnatally, indicating that the requirement of motoneurons for these factors changes during development, and that postnatal survival is also controlled by neurotrophic factors (Sendtner et al, 2000).

Neurotrophic support, mainly provided by target-tissue, regulates motoneuron survival throughout the physiological process of the developmental motoneuron cell death. During the development of higher vertebrates, motoneurons are generated in excess. In the lumbar spine of the developing rat, about 6000 motoneurons are present at embryonic day 14. These neurons grow axons that establish contact with the skeletal muscle, but about 50% of the motoneurons are lost during a critical period from embryonic day 14 until postnatal day 3 (Sendtner et al, 2000). This cell death is thought to be initiated if the neuron does not receive an adequate amount of neurotrophic support from its postsynaptic tissue during this crucial time period (Wetts et al, 1998).

Moreover, it is very likely that these factors influence specific properties of motoneurons, such as the regulation of transmitter synthesis and release, axon outgrowth and dendrite arborization as well as synaptic stability.

4.2.2.4. Necrosis and apoptosis

Cell death is a very important process in maintaining adequate cell numbers and functional integrity. Cell death occurs either via necrosis or apoptosis.

Necrosis generally occurs in pathology or following a severe and sudden injury that compromises the cell's homeostatic mechanisms. It is characterized by rapid cell disintegration, cellular swelling, organelle dysfunction, and passive cell disassembly. These events activate the immune system, leading to macrophage invasion and local inflammation.

In contrast, apoptosis is characteristic of normal tissue turnover and developmental cell death; therefore, it is considered to be a physiological process. It is an active process requiring

energy consumption and is accomplished by specialized and highly conserved cellular mechanisms that lead to morphological alterations. Apoptosis is characterized by early chromatin condensation, followed by internucleosomal DNA cleavage, cell shrinkage, reorganization of the cytoskeleton, organelle relocation and production of apoptotic bodies. Apoptosis requires the activation of certain genes and is carried out by a family of cysteine proteases called caspases, especially caspase-3 (Li et al, 1998b).

In the nervous system, cell death may involve aspects of both apoptosis and necrosis (Lawson et al, 1998; Oliveira et al, 2002). Moreover, there is evidence that motoneuronal cell death during development is apoptotic in origin (Hori et al, 2002). In a model of an incomplete midthoracic injury in the adult rat, neuronal loss was accompanied by a loss of glial cells. While acute motoneuron loss seemed attributable to necrosis, glial loss was associated with both necrosis and apoptosis (Grossman et al, 2001).

These results and others indicate that the pathway of degeneration in the nervous system is complex and probably differs depending on species, age, neuronal phenotype, and type of lesion or injury. Accordingly, caution must be exercised in attempts to strictly distinguish apoptotic vs. necrotic pathways, since neuronal death may overlap and vary along an apoptosis-necrosis continuum (Li et al, 1998a).

Taken together, the early loss of ventral horn motoneurons observed in our slice cultures correlates well with the observation that immature motoneurons are vulnerable to target deprivation. Moreover, this loss of a defined neuronal population seems to follow a general reaction pattern of “organotypic” slice culture preparations: Even in the well-established entorhinal-hippocampal slice culture model, in which defined hippocampal neuronal populations persist, great neuronal loss is observed in adjacent cortical regions.

4.3. REACTION PATTERNS OF GLIAL POPULATIONS

4.3.1. Astrocytic reaction and glial scar formation after injury

GFAP-immunoreactivity in our SCSC model follows an interesting spatiotemporal pattern. While in immediately fixed slices it is found almost exclusively in the white matter, reactive astrocytes begin to appear in the gray matter after 3 div, and GFAP immunoreactivity remains elevated at later time points. Our observations are in accordance with *in vivo* experiments, since slice culture preparation bears close resemblance to spinal cord transection models regarding glial cell reactions:

In vivo, after a C2 spinal cord hemisection, changes in astrocyte morphology and increase in GFAP-immunoreactivity were observed near the site of the lesion within one hr and persisted with no further significant morphological changes at least for 24h (Hadley et al, 1997). In a spinal cord transection study, there was an upregulation of GFAP mRNA within 30 min that

persisted up to three days after the injury, while the increase of GFAP-immunoreactivity peaked after three days and remained high in both gray and white matter after one week (Morin-Richaud et al, 1998). Similar findings were described in a root avulsion and an axotomy model: GFAP-immunoreactivity was upregulated within a few hours after lesion, increased up to 48 h and remained elevated even after 14d (Nomura et al, 2001).

These and other studies suggest that spinal cord astrocytes are extremely sensitive to both major as well as minor alterations of their microenvironment. Rapid changes in astroglial morphology, as detected by altered GFAP-immunoreactivity, may play a role in changes in neuronal function following SCI.

4.3.1.1. Two-phase increase in GFAP-immunoreactivity

It is proposed that this increase of GFAP-immunoreactivity in reactive astrocytes has two phases:

First, there is an early increase in GFAP-immunoreactivity without apparent increase in the protein level. Alterations in the surrounding microenvironment may result in depolymerization and redistribution of GFAP, thus altering the astrocyte form (process retraction) and the intensity of GFAP-immunoreactivity through exposure of more antigenic sites available for antibody binding.

Indeed, it is well known that CNS injuries cause an increase in anaerobic glycolysis, resulting in accumulation of lactate and acidosis. Acidic pH was shown to rapidly increase GFAP-immunoreactivity in cultured astrocytes (Oh et al, 1995). Another possible stimulus for this GFAP depolymerization following SCI is the rapid and dramatic increase (10- to 30-fold within minutes after injury) in extracellular potassium concentrations, which results in a further decrease of the intracellular pH. Upon depolymerization, GFAP may be redistributed towards the astroglial soma during the process of astrocyte process retraction, resulting in altered GFAP-immunoreactivity and the appearance of thickened and shortened astroglial processes.

In the second phase, reactive astrocytes begin to synthesize new GFAP protein resulting in increased GFAP-immunoreactivity at later time points of the astrocytic response (Hadley et al, 1997).

4.3.1.2. Reactive astrogliosis

This upregulation of GFAP-immunoreactivity is one of the hallmarks of “reactive astrogliosis” or “glial scarring”. Reactive astrogliosis is the relatively uniform CNS response to a variety of harmful stimuli such as trauma or ischemia and has been suggested to be an attempt at restoring homeostasis through isolation of the damaged region. Although other resident cells (i.e. microglia, oligodendrocytes, meningeal cells and stem cells) are also involved in the initial stages, the end result is a predominantly astrocytic scar tissue consisting of tightly packed, hyperfilamentous astrocytes, with many of their processes tightly apposed

to one another with limited extracellular space and with many gap and tight junctions between them. Astrocytes close to the lesion upregulate vimentin and nestin production, and cell division is seen (Fawcett et al, 1999).

This dense plexus of astroglial cells is often referred to as anisomorphic gliosis, whereas isomorphic gliosis refers to reactive astrocytes that have yet to form an interdigitating wall. Isomorphic gliosis is characterized by increased astrocyte hypertrophy and after neurotrauma may be a transient response prior to glial scar formation (McGraw et al, 2001). Astrocyte processes make up much of the bulk of the normal CNS parenchyma, and in response to injury there is proliferation of astrocytes and an increase in the size and complexity of their processes.

4.3.1.3. The inhibitory effect of reactive astrogliosis on axonal regeneration

A major cause of the failure of axon regeneration in the CNS is the inhibitory nature of this glial environment. However, the regenerative ability of most CNS axons is very low, so even fairly minor degrees of inhibition are probably sufficient to prevent regeneration. All cell types involved in the scar have inhibitory properties, and inhibition mediated by anyone of them is probably sufficient to block regeneration of most CNS axons.

Reactive astrocytes produce many secreted and cell surface molecules that could render astrocytes inhibitory to axonal regeneration. Among them, chondroitin sulphate proteoglycans, especially neurocan and brevican, are the most important inhibitory molecules. Furthermore, the three-dimensional tight astrocytic plexus of the glial scare, may pose a mechanical obstacle to regenerating axons (Fawcett et al, 1999).

4.3.1.4. The growth-promoting properties of astrocytes

However, not all cells are inhibitory all the time. Astrocytes in particular are enormously plastic, and can display growth-promoting or growth-inhibiting properties under different circumstances and in response to different stimuli (Fawcett et al, 1999). After a dorsal hemisection, cultured cortical neonatal rat astrocytes were transplanted into the lesion with collagen as a vehicle. After one month, histological data showed that the prelabelled astroglial cells survived transplantation and were localized predominantly in the collagen implant. Virtually no GFAP-positive astroglial cells migrated out of the implant into the adjacent host spinal cord. The presence of transplanted neonatal astroglial cells resulted in a significant increase in the number of ingrowing neurofilament-positive fibers (including anterogradely labeled corticospinal axons) into the implant, compared to collagen-only-implants controls. Ingrowing fibers were closely associated with the transplanted astroglial cells. The implantation of neonatal astroglial cells did result in modest temporary improvements of locomotor recovery as observed during open-field locomotion analysis or during crossing of a walkway (Joosten et al, 2004). These results indicate that astrocytes may be beneficial for neuronal regeneration and promote recovery after SCI.

The greater regenerative capacity after CNS injury by neonatal animals compared to adults could be partially attributed to the fact that the spread and the intensity of reactive gliosis are much greater among the latter (Ridet et al, 1997).

4.3.2. The role of microglial cells

Microglial cells are resident CNS macrophages that play an important role after injury. Immediately fixed spinal cord slices contain a very limited number of IB_4^+ cells. The majority of these are seen close to the remnants of spinal capillaries, possibly representing perivascular microglia. Very few "resting", ramified cells and some cells with an ovoid or amoeboid soma and short processes are found scattered across the gray and white matter with no specific distribution pattern, which is in accordance with earlier observations (Stoll et al, 1999). After 3 div there is a striking increase in the number of microglial cells at sites of focal tissue destruction, the white matter and mainly the dorsal corticospinal tract, reflecting the clearance of degenerating descending axons by activated microglia. The number of microglial cells remains elevated after 6 div, but their number seems to decrease after prolonged culture periods (9-12 div).

Microglia, the resident macrophages of the CNS, contribute to ca 10% of the total glial population in the adult CNS. They are located in close vicinity to neurons in the gray matter and between fiber tracts in the white matter of the CNS (Stoll et al, 1999). It is well known that following CNS injury a rapid activation and proliferation of microglial cells occurs, but microglial cell numbers and degree of microglial activation decrease sometimes even to control levels with time (Hailer et al, 1999; Rabchevsky et al, 1997). A study involving two models of peripheral nerve injury indicated that apoptotic cell death is one mechanism by which activated microglia are gradually eliminated following CNS injury and steady state of microglial cell number is achieved *in vivo* (Liu et al, 1997; Shuman et al, 1997).

4.3.2.1. Microglial subpopulations

It has been proposed that microglia/macrophages in the CNS can be divided into several subtypes based on their localization and morphology: parenchymal microglia, perivascular microglia and leptomeningeal macrophages. Perivascular microglia represent the only population of constitutive macrophages of the CNS, whereas parenchymal microglia represent a population of facultative phagocytes (Streit et al, 1999).

Parenchymal microglia differentiate from monocytes that migrate into the CNS early during embryonic development and are maintained as a pool with a low turnover rate. Perivascular microglia are in close contact to blood vessels and constitute a separate group with a higher turnover rate than parenchymal microglia. This pool is thought to be regularly replenished by monocytes from the peripheral circulation, which have infiltrated the CNS and may be responsible for the initial contact with an invading microorganism. An occasional

perivascular microglial cell may penetrate the CNS and differentiate into parenchymal microglia, but the use of bone marrow-chimeric animals has indicated that this sequence is a rare event (Gonzalez-Scarano et al, 1999).

4.3.2.2. The concept of microglial functional plasticity

The concept of microglial functional plasticity describes the ability of microglial cells to acquire different phenotypes dependent on their state of activation. In their resting state microglial cells demonstrate a ramified morphology. When activated in the adult CNS, microglial cells may transform from the ramified to the amoeboid, macrophage-like morphology. Activated microglial cells develop enlarged cell processes, which give the cells a bushy appearance.

Concomitant with cellular hypertrophy there is an upregulation of constitutively expressed CR3 complement receptors on the microglial cell surface, whereas an enhanced expression of MHC antigens becomes apparent at a later time point (Streit et al, 1999).

In the developing CNS this transformation takes a reverse course. In the developing CNS primordial microglia have a rounded or amoeboid morphology and as the CNS matures through the fetal and early postnatal period, these amoeboid microglia differentiate into increasingly ramified cells. There are a number of observations indicating that the macrophage-like appearance of primitive microglia has nothing to do with phagocytic activity and it may be a reflection of immaturity (Streit et al, 1999).

Our observation that most microglial cells found in acutely fixed slices display an amoeboid morphology may reflect the immature state of these cells in cultures derived from animals 12 days of age or younger.

4.3.2.3. Microglial activation at the site of neuronal or axonal damage

At the site of inflammation activated microglia become phagocytic. Microglia release inflammatory cytokines that amplify the inflammatory response by recruiting cells to the injury site. In addition, microglia (like macrophages) can release a variety of potentially harmful soluble factors, including oxygen and nitrogen intermediates, proteolytic enzymes, arachidonic acid metabolites and proinflammatory, potentially cytotoxic cytokines such as tumor necrosis factor (TNF) α or interleukin (IL)-1 (Gonzalez-Scarano et al, 1999; Stoll et al, 1999).

4.3.2.4. The spatiotemporal course of microglial activation after injury

Following injury, cellular hypertrophy of microglial cells is apparent after 24 hrs. Microglial cells begin to proliferate 2-3 days after injury, their numbers reach maximal levels after 4-7 days, and usually subside at later time points, since microglial activation after acute CNS injury is a transient and self-limiting event. This pattern has been described in most experimental models: In an entorhinal cortex lesion model in adult rats microglia counts and

the proliferation rate in the ipsilateral dentate gyrus reached a maximum in the molecular layers at 3 days post-lesion (dpl) and returned to control levels by 30 dpl (Hailer et al, 1999). After dorsal hemisection of the rat spinal cord, proliferation and recruitment of macrophages and microglial cells becomes predominant 2 days after injury, and their density is at its highest at the lesion site between 4 and 8 days (Dusart et al, 1994). Glial proliferation induced by dorsal root lesion and sciatic nerve injury begins at 1 day with peaks from 2-4 days postoperatively (Liu et al, 2000). In spinal contusion models, microglial cell activation displayed a similar temporal pattern (Carlson et al, 1998; Koshinaga et al, 1995; Popovich et al, 1997).

In most spinal cord lesion models upregulation of microglial activity was not confined to the lesion site. Microglial activation distal to the lesion may contribute to neuronal trophism and plasticity in the lesioned spinal cord (Carlson et al, 1998; Leme et al, 2001). In a comparative experimental model, the extent and severity of the inflammatory reaction in the spinal cord greatly exceeded that seen in the brain at all time points analyzed (Schnell et al, 1999) .

Taken together, microglial activation is an early, important step of the CNS reaction to acute injury, involving several non-neuronal cell populations (neutrophils, microglia/macrophages, oligodendrocytes, astrocytes).

In SCSC, spatiotemporal activity pattern of the IB_4-immunoreactive cells largely correlates with that found *in vivo*. The increased IB_4-immunoreactivity and the microglial cell morphology observed in the white matter and especially in the dorsal corticospinal tract reflect microglial phagocytic activity, initiated by demyelination and degeneration of axons belonging to the interrupted spinal tracts. This reaction remains limited, affecting defined regions and subsiding after some time.

4.4. NESTIN AND STEM CELLS IN THE INJURED SPINAL CORD

4.4.1. Nestin expression in the spinal cord

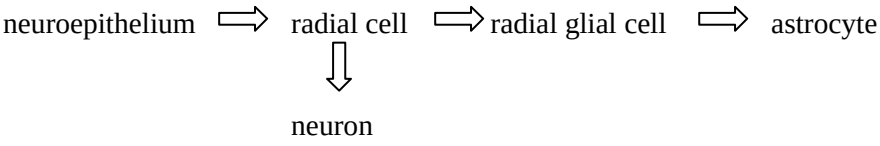
In acutely fixed slices, nestin-immunoreactivity displays a pattern similar to GFAP-immunoreactivity in the white and gray matter, while in an area around the central canal it resembles the distribution of CB-immunoreactivity. In acutely fixed slices from p0 to p6 animals nestin immunoreactivity was observed mainly in the subependymal zone, the dorsomedial septum and the ventral comissure, and this is in accordance with nestin expression described by Moreels (Moreels et al, 2005).

Nestin is a cytoskeletal embryonic intermediate filament that appears prenatally in immature cells of neuroepithelial origin (Kalman et al, 2001). Nestin is downregulated either at the onset of GFAP or neurofilament expression, or during subsequent differentiation of multipotential neural precursors into astrocytes or neurons. The transient abundant expression

of nestin is considered a marker for neural precursor cells in the developing CNS of mammals (Wei et al, 2002). In postnatal rat spinal cord the nestin gene was found to be downregulated after postnatal day 6 (Dahlstrand et al, 1995; Schmidt-Kastner et al, 2002). This is in accordance with our observation of decreased nestin expression in cultures derived from p9 or p12 animals. In the adult nervous system nestin expression is downregulated and very low expression levels are observed except in the choroid plexus, in certain Schwann cells and in endothelial cells (Frisen et al, 1995).

These nestin$^+$ cells are described to be radial glial cells. Radial glial cells are transient cells in the developing CNS, which are known to be involved in guiding migrating neurons (Moreels et al, 2005). Postnatally they transform into astrocytes. Radial glial cells share molecular similarities both with neuroepithelial cells and astrocytes. Primate radial glial cells express both nestin and GFAP, whereas in rodents, they express nestin but no GFAP.

In the developing rat spinal cord, tracing experiments with specific neuroepithelial and gliogenic markers have revealed heterogeneity among nestin$^+$ precursor cells and suggest a lineage progression from neuroepithelial cells through to astrocytes in the developing spinal cord:



Radial glial cells represent the earliest appearance of a glial-committed phenotype. The following transformation from radial glial cells to astrocytes clearly involves cell proliferation. Few if any neurons appear to be derived from radial glial cells, which are instead the major sources of astrocytes in the spinal cord (Barry et al, 2005).

Nestin activity is downregulated in all regions of the spinal cord by p6, but these nestin$^+$ cells are thought to be already committed to a glial lineage, and therefore nestin activity cannot be considered as a marker for neuronal progenitor cell potential in the postnatal rat spinal cord. In conclusion, in our model, nestin$^+$ cells in acutely fixed slices seem to represent radial glial cells that are already committed to the glial lineage and are downregulated in slices derived from animals older than p6.

4.4.2. Nestin upregulation and the role of stem cells after SCI

The time pattern of nestin immunoreactivity upregulation is similar in most injury models: It starts during the first 24 hrs, reaches a peak after 7 days, remains elevated, and decreases slowly up to the 4th week (Liu et al, 2002; Sahin et al, 1999; Shibuya et al, 2002). On the contrary, in an organotypic slice culture model of cortex of p10 rats, there is no evidence of downregulation of nestin even 8 weeks after explantation. Since nestin-immunoreactivity is absent from glial cells in sections of the cortex of fully mature rats, these findings indicate a different course of glial maturation *in vivo* and *in vitro*. This absence of nestin-

downregulation may be due to the absence of certain stimuli in the slice culture which otherwise lead to the physiological switch in intermediate filament protein expression in glial cells *in vivo*. Another explanation could be that nestin expression reflects the complex influence of the condition of slice culture, in which the nutrition occurs through the extracellular space, leading to a more immature phenotype for astrocytes (Schmidt-Kastner et al, 2002).

Recent experiments with adult rats suggested that a stem cell population that divides as early as 24h after SCI is localized throughout the spinal cord. This robust proliferation leads to the production of new glial cells involved in astrogliosis and the generation of new myelin. Neuronal phenotypes are not generated following SCI (Horky et al, 2006). Following SCI in primates, robust endogenous cell division leads to the formation of oligodendrocytes and to the generation of new astrocytes. New neuron formation derived from cells dividing within 5d of injury was not observed (Yang et al, 2006). Taken together there is increasing evidence that the spinal cord possesses stem cells that are able to multiply and differentiate after injury. These cells seem to be already committed to the glial lineage, since they differentiate into astrocytes and oligodendrocytes. There is no evidence for new neuron formation following SCI.

In SCSC derived from p0, p3 or p6 animals nestin expression was elevated compared to preparations from p9 or p12 animals. The initial pattern of nestin immunoreactivity is in accordance with the presence of neural stem cells in the ependymal layer and the subpial astrocyte layer, as these are the sites of the major nestin immunoreactivity. It also illustrates the plasticity of young postnatal astrocytes that maintain high nestin levels. The similar pattern of nestin-, and GFAP-immunoreactivity observed in our slice culture model in acutely fixed slices reflects the coexistence of $nestin^+$ and $GFAP^-$ radial glial cells with $GFAP^+$ astrocytes. Moreover, the pattern of nestin upregulation in our culture model at later time points and the re-expression of nestin in cultures derived from older animals reflects the nestin expression upregulation in reactive astrocytes and the proliferation of stem cells existing in the spinal cord of animals of any age. These findings are in accordance with the *in vivo* models of SCI.

PART II: ESTABLISHING A SLICE CO-CULTURE MODEL OF MOTORCORTEX AND SPINAL CORD

In the second part of this study we set out to establish a practicable model that enables the investigation of axonal sprouting under various experimental conditions. We therefore combined slice cultures of rat motorcortex and spinal cord with anterograde axonal labeling.

This co-culture system was then applied to investigate effects of pia and white matter removal and of exogenously administered NT-3 on neuronal survival and axonal sprouting.

4.5. DEATH OF CORTICOSPINAL NEURONS AFTER LESION. THE ROLE OF AGE AND TARGET-DEPENDENCY

We found that the number of surviving cortical pyramidal neurons sharply declined in the control group after 4 div. The ability of corticospinal neurons to survive axotomy appears to be temporally well correlated with their innervation of spinal targets. These neurons die if their axons are cut prior to target innervation, but are able to survive if axotomy occurs after their axons have innervated spinal targets. Axotomy after 14 days or later caused cell shrinkage but not cell death (Merline et al, 1990; Tolbert et al, 1987).

A possible explanation for the difference between developing and mature neurons may lie in a greater dependence on target-derived neurotrophic support for the developing neurons. Axotomy prevents the trophic factors from reaching the cell body, and the neurons subsequently die. In contrast, mature neurons may possess sufficient alternative sources of support (Korsching, 1993).

It was also reported that axotomy of corticospinal axons in the adult rat, close to the perikarya, i.e. at the level of the internal capsule, resulted in cell death of about 50% of the corticospinal neurons (Giehl et al, 1996). Axotomy close to the cell body even of mature neurons seems to deprive the neuron of a proportion of its collaterals that is larger than the minimum required for survival, thus leading to retrograde cell death (Bregman et al, 1998).

Since in our co-culture model slice preparation equals a severe proximal axotomy and the cultures are derived from p4 animals, the observed pyramidal cell death in the control group is an expected finding. These findings bear similarities to the early death of spinal cord motoneurons previously described in our SCSC model.

4.6. THE ROLE OF EXOGENOUS NT-3 APPLICATION

4.6.1. The neuroprotective effect of NT-3

Prevention of retrograde degeneration in the injured spinal tracts is an important prerequisite for successful axonal regrowth, because functional restoration will not occur if the neurons have died. The improved survival of cortical neurons and enhanced preservation of the cortical cytoarchitecture in the NT-3 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 (Giehl et al, 1996; Hammond et al, 1999; Novikova et al, 2002).

There is evidence indicating the existence of optimal time intervals for exogenous neurotrophin administration. It is known that the efficacy of neurotrophic factors decreases with increasing dose or increasing duration of treatment. This may be partially due to desensitization of the receptors, or to adverse cumulative effects of neurotrophins (Novikova et al, 2000). In our experimental model NT-3 displayed a significant neuroprotective effect. Our treatment protocol was relatively short (4 div) and NT-3 administration began immediately after co-culture preparation. Further experiments are required in order to define the optimal time pattern of NT-3 administration.

4.6.2. NT-3 promotes axonal regeneration and sprouting

In the NT-3 group, labeled corticospinal sprouting axons with a maximal length of 500µm were observed in the corticospinal interface, indicating an effect of NT-3 on stimulating axonal regrowth and inducing axonal sprouting.

In vivo, NT-3 has been found to enhance regeneration and sprouting of corticospinal axons both during development and after adult spinal cord lesion (Schnell et al, 1994). Similar results were observed in experiments where NT-3 was delivered through NT-3 producing fibroblasts grafts (Arvanian et al, 2003; Grill et al, 1997), collagen containing NT-3 (Houweling et al, 1998), or where NT-3 was combined with fetal spinal cord transplants (Bregman et al, 1997), intercostal nerve implants (Blits et al, 2000), or antibodies neutralizing the myelin-associated neurite growth inhibitors (von Meyenburg et al, 1998).

Lately, much research has been focused on gene transfer with adeno-associated viral vectors encoding these neurotrophic factors (Koda et al, 2004; Tai et al, 2003; Tang et al, 2004).

4.7. MYELIN-ASSOCIATED GROWTH-INHIBITORS

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, indicating that the regenerating axons try to avoid the white matter.

This observation underlines the relevance of myelin-associated growth inhibitors and is in accordance with *in vivo* findings: *In vivo* experiments using grafts or various substrates at the lesion site have shown that regenerating and sprouting axons are found almost exclusively within the remaining gray matter at the lesion area, mainly avoiding the host white matter. When injured corticospinal axons were presented with a choice between using the graft, the

host white matter, or the host gray matter as a growth substrate, they used only the host gray matter (Blesch et al, 1999; Blits et al, 2000; Ferguson et al, 2001; Grill et al, 1997; von Meyenburg et al, 1998).

This preference for the host gray matter may be associated with the myelin-associated growth inhibitors. Many proteins present in CNS myelin, Nogo (Nogo-A, -B, and –C isoforms), myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp), are capable of causing growth cone collapse and inhibit neurite outgrowth *in vitro*. The main isoform Nogo-A is predominantly expressed in the nervous system with lower levels also present in testis and heart. In CNS myelin, Nogo-A is expressed in oligodendrocyte cell bodies and processes surrounding myelinated axons and is localized in the innermost adaxonal and outermost myelin membranes. These cell-surface proteins signal through multi-subunit neuronal receptors that contain a common ligand-binding glycosylphosphatidylinositol-anchored subunit termed the Nogo-66 receptor (NgR) (Niederost et al, 2002).

The NgR protein is detected selectively in neurons and is present throughout axons, indicating that Nogo-A and its receptor are juxtaposed along the course of myelinated fibers. NgR protein expression is restricted to postnatal neurons and their axons (Huber et al, 2002).

After SCI, Nogo-A is upregulated to a moderate degree, whereas NgR levels are maintained at constant levels. In the injured spinal cord, interactions between NgR and its ligands are most likely to be important for limiting regeneration of corticospinal and some other descending tracts; other receptors may be more important for ascending tracts (Huber et al, 2002; Wang et al, 2002).

In order to investigate the role of myelin-associated axonal-growth inhibitors, mice with a Nogo mutation that eliminates Nogo-A/B expression were analyzed. Numerous fibers regenerated into distal cord segments of Nogo-A/B(-/-) mice and recovery of locomotor function was also improved, indicating that Nogo-A plays a key role in restricting axonal sprouting in the young adult CNS after injury (Kim et al, 2003).

When intact adult rats were treated with the IN-1 antibody, an antibody capable of binding and neutralizing Nogo-A, they exhibited an increase of aberrant cortical neurons' projections, i.e., sensory fibers projecting into the ventral horn and motor fibers projecting dorsally. Unilateral pyramidotomy in the presence of the IN-1 monoclonal antibody triggered a progressive reorganization of the sprouting of the remaining corticospinal fibers across the midline, with sensory fibers projecting gradually into the denervated dorsal horn and motor fibers projecting into the denervated ventral horn (Bareyre et al, 2002).

To evaluate whether neutralizing the neurite growth inhibitor Nogo-A after spinal lesion, promotes axonal sprouting and functional recovery in primates, 12 monkeys were subjected to unilateral cervical lesion (C7-C8 level). Recovery of manual dexterity and sprouting of corticospinal axons caudal to the lesion were enhanced in monkeys treated with anti-Nogo-A-specific antibody as compared to monkeys treated with control antibody (Freund et al, 2006).

Moreover, anterograde labeling of the interrupted corticospinal tract showed that retrograde axonal degeneration was lower in anti-Nogo-A antibody treated monkeys. Treatment with the anti-Nogo-A antibody also enhanced axonal sprouting rostral to the cervical lesion, with some of these fibres growing around the lesion and into the caudal spinal segments. These processes paralleled the observed improved functional recovery (Freund et al, 2007).

Taken together, it appears that the presence of an adequate, growth-supportive environment is essential for neurotrophin-induced axonal sprouting in order to achieve reinnervation of target tissues.

4.8. OVERVIEW

In summary, the following hypotheses were examined in this study:

Hypothesis 1: In the first part of our study we examined the hypothesis that an organotypic slice culture of postnatal rat spinal cord is a functional *in vitro* model where defined neuronal and glial populations remain preserved over longer culture periods.

We here provide evidence that *in vitro* culture of postnatal rat spinal cord is possible, as defined neuronal and glial populations remain well preserved over a culture period of at least 12 div.

Hypothesis 1a: We tested the hypothesis that tissue preservation of spinal cord OSCs is influenced by two important parameters: *i)* the animal age at the time of explantation, and *ii)* the duration of the *in vitro* period.

We found that the immunohistochemical aspect of the acutely fixed preparations revealed no significant differences among various age groups, with the exception of nestin-immunoreactivity, which was elevated in preparations derived from younger animals (p0-p6), compared to those derived from older ones (p9-p12). Moreover, cultures derived from younger animals (p0 or p3) appeared to be better preserved than those derived from older animals (p9 or p12). With prolonged culture time there was a gradual degradation in tissue quality and cytoarchitecture preservation. The most prominent alterations, however, occurred during the first three days *in vitro*, with the appearance of the various cellular populations remaining generally unchanged afterwards.

Hypothesis 1b: We further hypothesized that some degree of degenerative changes in defined neuronal populations and glial activation is to be expected, since interruption of all afferent and efferent neuronal pathways is unavoidable during slice culture preparation.

The preservation of distinct neuronal populations in our SCSCs indicates that some populations are little affected by the interruption of most afferent and efferent neuronal pathways, since local neuronal circuits and cell-to-cell interactions remain preserved inside the slice cultures. On the other hand, the early loss of $ChAT^+$ motoneurons and other large $NeuN^+$ and CB^+ neurons shows that SCSC cannot be considered “organotypic” in all aspects.

This is not an entirely unexpected finding, since these cells are target-dependent for survival through postnatal age. The reaction of glial cells reveals that their activation is limited in both time and space.

Finally, we compared our findings to well established *in vivo* findings and show that findings *in vitro* are similar to *in vivo* findings in SCI models, since our SCSC model bears close resemblance to SCI models.

It seems reasonable to propose that our model of SCSC could prove useful in future experiments on the pathophysiology of SCI, although some limitations have to be observed, as in other *in vitro* models.

Having established and characterized the postnatal rat SCSC model we established an *in vitro* slice co-culture model of SCSCs with cortical slice cultures. The aim of these co-cultures was to investigate whether corticospinal sprouting occurs, and whether *i)* neurotrophic factors, and *ii)* white matter removal influence this process.

Hypothesis 2: We tested the hypothesis that cultures of cerebral cortex adjacent to spinal cord explants should enable outgrowth of CST axons.

We provide evidence that the effects of exogenous administration of neurotrophic or anti-repellent factors on neuronal regeneration in the corticospinal system can easily be investigated, because neurons survive and axonal regeneration and sprouting occur in our model.

Hypotheses 2a, b: We further tested the hypotheses that *i)* exogenous NT-3 administration has the potential to improve neuronal survival and to promote axonal regrowth *in vitro,* while *ii)* spinal cord white matter inhibits axonal outgrowth and penetration into spinal cord gray matter.

We found that NT-3 enhances the survival of corticospinal neurons and prevents neuronal cell death; furthermore, that it promotes regenerative axon elongation and sprouting. White matter interposition seems to inhibit axonal penetration into the spinal gray matter.

These *in vitro* observations are in accordance with *in vivo* experimental findings. This model will hopefully become a useful tool in future experiments that aim at elucidating the complex effects of neurotrophic factors and white matter on neuronal preservation, axonal regenerating, and sprouting after SCI.

5. SUMMARY

Spinal cord injury induces degenerative and regenerative processes and complex interactions of neurons with non-neuronal cells. The injured spinal cord responds to neurotrophin application with enhanced neuronal survival and axonal regeneration.

In order to provide an *in vitro* tool for the investigation of such processes we prepared spinal cord slice cultures (SCSC) from Wistar rats (p0-12). SCSC were sustained *in vitro* up to 12 days and characterized by immunohistochemistry. Calbindin$^+$ neurons, distributed across the entire gray matter, were visible also after longer culture periods. NeuN$^+$ neurons were best preserved in the dorsal horn, whereas large NeuN$^+$ and ChAT$^+$ motoneurons in the ventral horn vanished after 3 days *in vitro*. GFAP$^+$ astrocytes, initially restricted to the white matter, invaded the gray matter of SCSC early during the culture period. Microglial cells, stained by *Griffonia simplicifolia* isolectin B_4, were rapidly activated in the dorsal tract and in the gray matter, but declined in number with time. Nestin-immunoreactivity was found in animals of all age groups, either in cells interspersed in the ependymal lining around the central canal, or in cells resembling protoplasmic astrocytes. SCSC derived from p0 or p3 animals showed a better preservation of the cytoarchitecture than cultures derived from older animals. In summary, SCSC contain defined neuronal populations, the cytoarchitecture is partially preserved, and the glial reaction is self-limited.

In the second part of our study, we investigated the effects of exogenous neurotrophin (NT)-3 and white matter removal on regenerating corticospinal axons by using co-cultures of motorcortex and spinal cord slice culture, obtained from p4 Wistar rats. Motorcortex pyramidal neurons were anterogradely labeled with Mini-Ruby, a 10kD biotinylated dextran amine. Cultures were divided into two groups; one control group cultured with culture medium and the NT-3 group, where NT-3 (50 ng/ml) was added to the culture medium. The NT-3 group was further divided into two subgroups; a “whole slice” subgroup and a “half slice” subgroup, where there was no spinal cord white matter interposition between cortical and spinal gray matter. This was achieved by the fact that spinal cord slices were cut into halves, thus enabling the direct contact of cortical and spinal grey matter. In contrast to the control group, the group of co-cultures treated with NT-3 showed an improved survival of cortical neurons, and contained labeled, regenerating corticospinal axons. These sprouting axons entered the adjacent spinal cord tissue in the “half slice” subgroup, while they were unable to cross the corticospinal interface in the “whole slice” subgroup. Our data suggest that exogenous NT-3 administration has the potential to improve neuronal survival and to promote axonal regrowth *in vitro*, while white matter interposition exhibits inhibitory effects on axonal regeneration and penetration into spinal cord gray matter. The co-culture model of motorcortex with spinal cord provides an interesting experimental paradigm for the investigation of regenerative processes following spinal cord injury.

6. ZUSAMMENFASSUNG

Die akute Verletzung des Rückenmarkes löst degenerative und regenerative Prozesse sowie komplexe Interaktionen von Neuronen mit nicht-neuronalen Zellen aus. Diese Prozesse sind noch weitgehend unverstanden, und es ist nach wie vor nicht möglich, eine erfolgreiche Regeneration des verletzten Rückenmarkes zu induzieren. Ziel dieser Arbeit war es, ein *in vitro* Modell zur besseren Erforschung degenerativer und regenerativer Prozesse des verletzten Rückenmarkes zu entwickeln. In einem ersten Schritt wurden Schnittkulturen des Rückenmarkes der Ratte angefertigt und charakterisiert. Im zweiten Schritt wurden Ko-Kulturen von Rückenmark und sensomotorischem Kortex angelegt, in denen regenerative Vorgänge untersucht werden sollten.

Um ein *in vitro* Modell zu entwickeln, wurden Rückenmarkschnittkulturen von Wistar-Ratten (p0-12) hergestellt. Diese Schnittkulturen wurden *in vitro* bis zu 12 Tage kultiviert und immunhistochemisch charakterisiert. Calbindin$^+$ Neurone waren in der gesamten grauen Substanz auch nach längeren Kultivierungszeiten zu sehen. NeuN$^+$ Neurone waren im Hinterhorn besser erhalten, während eine große Zahl von NeuN$^+$ und Cholin-Azetyl-Transferase (ChAT)$^+$ Motoneuronen im Vorderhorn nach 3 Tagen *in vitro* verschwanden. Glial fibrillary acidic protein (GFAP)$^+$ Astrozyten, am Anfang nur auf die weiße Substanz begrenzt, waren nach kurzer Zeit auch in der grauen Substanz zu finden. Mikrogliazellen, gefärbt mit *Griffonia simplicifolia* Isolektin B_4, wurden schnell in den dorsalen Bahnen und in der grauen Substanz aktiviert, aber ihre Anzahl ging später zurück. Eine Nestin-Immunoreaktivität war in Tieren aller Altersgruppen zu finden, entweder in Ependymzellen um den Zentralkanal oder in protoplasmatischen Astrozyten. Schnittkulturen von p0 oder p3 Tieren zeigten eine bessere Erhaltung der Zytoarchitektur als Schnittkulturen von älteren Tieren (p6 bis p12). Zusammenfassend kann gesagt werden, dass Rückenmarkschnittkulturen definierte neuronale Populationen enthalten, die Zytoarchitektur teilweise erhalten ist, und dass die gliale Aktivierung mit der Zeit nach lässt.

Im zweiten Teil dieser Arbeit wurden die Effekte der exogenen Neurotrophin (NT)-3-Gabe und der Entfernung der weißen Substanz auf die Regeneration von kortikospinalen Axonen untersucht. Dazu wurden Ko-Kulturen von sensomotorischem Kortex und Rückenmark-Schnittkulturen von p4 Wistar Ratten verwendet. Neurone des sensomotorischen Kortex wurden anterograd mit Mini-Ruby, einem 10kD biotinylierten Dextranamin, markiert. Die Ko-Kulturen wurden in zwei Gruppen eingeteilt: eine Kontrollgruppe, die nur mit Kulturmedium kultiviert wurde, und die NT-3 Gruppe, in der NT-3 (50 ng/ml) dem Kulturmedium hinzugefügt wurde. Die NT-3 Gruppe wurde weiter in zwei Untergruppen unterteilt: eine Untergruppe mit „ganzen Schnitten“ und eine Untergruppe mit „halben Schnitten“. Bei letzterer wurde die Interposition von spinaler weißer Substanz zwischen kortikaler und spinaler grauen Substanz entfernt. Im Gegensatz zur Kontrollgruppe zeigte sich

in der mit NT-3 behandelten Gruppe ein verbessertes Überleben kortikaler Neurone, und es fanden sich mit Mini-Ruby markierte, regenerierende kortikospinale Axone. Diese regenerierenden Axone wuchsen in der Untergruppe „halber Schnitte“ in das anliegende Rückenmarkgewebe ein, während sie die kortikospinale Grenze in der Untergruppe „ganze Schnitte“ nicht überqueren konnten.

Die Daten zeigen, dass die Applikation von NT-3 das Potential hat, das neuronale Überleben zu verbessern und die axonale Regeneration zu fördern. Demgegenüber hat die Interposition von weisser Substanz inhibitorische Effekte auf die axonale Regeneration und auf das axonale Eindringen in die spinale graue Substanz. Das Ko-Kulturmodell von Schnittkulturen des sensomotorischem Kortex und des Rückenmarkes bietet ein interessantes experimentelles Modell für die Erforschung regenerativer Vorgänge nach einer Rückenmarkverletzung.

7. LIST OF ABBREVIATIONS

BDNF	**B**rain-**D**erived **N**eurotrophic **F**actor
BrdU	5-**Br**omo-2-**d**eoxy-**U**ridine
BSA	**B**ovine **S**erum **A**lbumin
CB	**C**al**B**indin-D28k
ChAT	**Ch**oline **A**cetyl**T**ransferase
CNS	**C**entral **N**ervous **S**ystem
CNTF	**C**iliary **N**euro**T**rophic **F**actor
CSN	**C**ortico**S**pinal **N**euron
CST	**C**ortico**S**pinal **T**ract
DAB	3, 3´-**D**i**A**mino**B**enzidine
GABA	**G**amma-**A**mino**B**utyric **A**cid
GAP-43	**G**rowth-**A**ssociated **P**rotein 43
GDNF	**G**lial-**D**erived **N**eurotrophic **F**actor
GFAP	**G**lial **F**ibrillary **A**cidic **P**rotein
HBSS	**H**ank's **B**alanced **S**alt **S**olution
HGF/SF	**H**epatocyte **G**rowth **F**actor/ **S**catter **F**actor
IB_4	**I**solectin **B_4**
IF	**I**ntermediate **F**ilament
IL-1	**I**nter**L**eukin **1**
LIF	**L**eukemia **I**nhibitory **F**actor
MAG	**M**yelin **A**ssociated **G**lycoprotein
MEM	**M**inimal **E**ssential **M**edium
MHC	**M**ajor **H**istocompatibility **C**omplex
MPSS	**M**ethyl**P**rednisolone **S**odium **S**uccinate
NeuN	**Neu**ronal **N**uclei
NGF	**N**erve **G**rowth **F**actor
NgR	**N**o**g**o-66 **R**eceptor
NHS	**N**ormal **H**orse **S**erum
NT-3	**N**euro**T**rophin-3
NT-4/5	**N**euro**T**rophin 4/5
OHSC	**O**rganotypic **H**ippocampal **S**lice **C**ulture
OMgp	**O**ligodendrocyte-**M**yelin **g**lyco**p**rotein
OSC	**O**rganotypic **S**lice **C**ulture
PB	**P**hosphate **B**uffer
PBS	**P**hosphate-**B**uffered **S**aline
PN	**P**eripheral **N**erve

PNS	**P**eripheral **N**ervous **S**ystem
SCI	**S**pinal **C**ord **I**njury
SCSC	**S**pinal **C**ord **S**lice **C**ulture
TNF-α	**T**umor **N**ecrosis **F**actor-α
trk	**t**y**r**osine **k**inase

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9. APPENDIX

9.1 ACKNOWLEDGEMENT

First of all I would like to express my deepest gratitude to my supervisor Priv.-Doz. Dr. med. Nils Peter Hailer for the assignment of this doctoral thesis as well as the promotion of my scientific activities.

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9.2 CURRICULUM VITAE

PERSONAL DETAILS

Name:	Stavros Stavridis
Date of birth:	7th May 1974
Place of birth:	Thessaloniki, Greece
Nationality:	Greek
Languages:	German, English, French

EDUCATION

1980-1986:	38th Primary school, Thessaloniki, Greece
1986-1992:	German School, Thessaloniki, Greece (Deutsche Schule, Thessaloniki) Graduated with an overall grade 19,10/11.

Medical

1992-1998:	Aristotle University of Thessaloniki
Qualifications:	Ptychio Iatrikes, 1998, Faculty of Medicine (Equivalent of Medical Degree) Graduated with an overall grade 8,7.

STUDENT ELECTIVES

Aug 1995:	N. Moudania Health Centre, Chalkidiki, Greece Training programme for medical students in emergency medicine
Aug 1996:	University Hospital of Essen, Germany Rotational training programme including two departments: Orthopaedics and Trauma Surgery

CURRENT EMPLOYMENT

Jan 2005-present:	Specialist Registrar in Orthopaedics A' Orthopaedic Department, Aristotle University of Thessaloniki "G. Papanikolaou" General Hospital, Thessaloniki, Greece

PREVIOUS EMPLOYMENT

Feb 2003-Feb 2004:	Compulsory military service as a trained Medical Officer in the Greek Army
Mar 2001-Feb 2003:	Research project in "Spinal Cord Compression" University Hospital for Orthopaedic Surgery Friedrichsheim; Institute of Anatomy II, Johann Wolfgang Goethe-University, Frankfurt a.M., Germany
Jan 2000-Oct 2000:	Senior House Officer Dept. of General Surgery "Ippokrateion" Hospital, Thessaloniki, Greece
Nov 1998-Nov 1999:	General practitioner residency, Kassandreia Health Centre, Chalkidiki, Greece
Aug 1998-Nov 1998:	Senior House Officer, General Hospital of Polygyros, Chalkidiki, Greece Rotational training programme including three departments: General Surgery, Internal Medicine, Cardiology

9.3 LEBENSLAUF

PERSÖNLICHE DATEN

Name:	Stavros Stavridis
Geboren am:	07.05.1974
Geburtsort:	Thessaloniki, Griechenland
Nationalität:	Griechisch
Fremdsprachen:	Deutsch, Englisch, Französisch

AUSBILDUNG

1980-1986:	38. Grundschule, Thessaloniki, Griechenland
1986-1992:	Deutsche Schule Thessaloniki, Griechenland (Abiturnote 1,0)

Universitäre Ausbildung

1992-1998:	Medizinstudium in der Aristoteles Universität von Thessaloniki
Qualifizierung:	Ptychio Iatrikis, 1998, (Diplom der Medizin) Abschlussnote: 8,7/10

FAMULATUREN

August 1995:	N. Moudania Gesundheitszentrum, Chalkidiki, Griechenland Ausbildungsprogramm für Medizinstudenten in Notfallmedizin
August 1996:	Universitätsklinikum Essen, Deutschland Orthopädie und Unfallchirurgie

AKTUELLE BESCHÄFTIGUNG

Jan 2005-heute:	Assistenzarzt in Orthopädie 1. Orthopädische Universitätsklinik, Aristoteles Universität von Thessaloniki „G. Papanikolaou“ Allgemeines Krankenhaus, Thessaloniki, Griechenland

VORHERIGE BESCHÄFTIGUNG

Feb 2003-Feb 2004:	Militärdienst als ausgebildeter Medizinoffizier in der griechischen Armee
Mar 2001-Feb 2003:	Forschungsprojekt „Spinale Kompression“ Orthopädische Universitätsklinik, Stiftung Friedrichsheim; Institut für Anatomie II, Johann Wolfgang Goethe-Universität, Frankfurt a. M., Deutschland
Jan 2000-Okt 2000:	Assistenzarzt in der Allgemeinchirurgie „Ippokrateion“ Allgemeines Krankenhaus, Thessaloniki, Griechenland
Nov 1998-Nov 1999:	Arzt im Kassandreia Gesundheitszentrum, Chalkidiki, Griechenland
Aug 1998-Nov 1998:	Assistenzarzt im Allgemeinen Krankenhaus in Polygyros, Chalkidiki, Griechenland, mit Rotation in Allgemeinchirurgie, Innere Medizin mit Kardiologie

9.4 HONORARY STATEMENT

With this I honorary declare that this work under the title

"Characterization of postnatal rat spinal cord slice cultures and studies on co-cultures of postnatal rat spinal cord and motorcortex"

which has been approved as a doctoral thesis in the faculty of human medicine of the Johann Wolfgang Goethe-University in Frankfurt a.M., has been completed by me under the supervision of Priv.-Doz. Dr. med. N. P. Hailer from the Orthopaedic University Hospital "Stiftung Friedrichsheim" and the support of Prof. Dr. med. H.-W. Korf from the Institute for Anatomy II of the "Dr. Senckenbergischen Anatomie" without any further assistance and that no other means than these mentioned in the dissertation have been used in the composition of this work.

Until now, I have neither applied for a doctoral thesis in any medical faculty inside or outside Germany, nor have I submitted the present work as Dissertation.

Parts of the present work were published as follow:

1: Original papers:

i) Stavridis SI, Dehghani F, Korf HW, Hailer NP.

Characterisation of transverse slice culture preparations of postnatal rat spinal cord: preservation of defined neuronal populations.

Histochem.Cell Biol. 2005 Jun;123(4-5):377-92.

ii) Stavridis SI, Dehghani F, Korf HW, Hailer NP.

Sprouting of corticospinal axons in co-cultures of rat motorcortex and spinal cord.

2008, submitted.

Frankfurt am Main,

_____

Stavros Stavridis

9.5 EHRENWÖRTLICHE ERKLÄRUNG

Hiermit erkläre ich ehrenwörtlich, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität in Frankfurt am Main zur Promotionsprüfung eingereichte Arbeit mit dem Titel

"Characterization of postnatal rat spinal cord slice cultures and studies on co-cultures of postnatal rat spinal cord and motorcortex"

unter der Leitung von Privatdozent Dr. med. N. P. Hailer von der Orthopädischen Universitätsklinik Stiftung Friedrichsheim und der Unterstützung von Prof. Dr. med. H.-W. Korf von dem Institut für Anatomie II der Dr. Senckenbergischen Anatomie ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.

Ich habe bisher an keiner Medizinischen Fakultät innerhalb oder außerhalb Deutschlands ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende Arbeit als Dissertation vorgelegt.

Teile der vorliegenden Arbeit wurden in folgenden Publikationsorganen veröffentlicht:

Originalarbeiten:

i) Stavridis SI, Dehghani F, Korf HW, Hailer NP.

Characterisation of transverse slice culture preparations of postnatal rat spinal cord: preservation of defined neuronal populations.

Histochem.Cell Biol. 2005 Jun;123(4-5):377-92.

ii) Stavridis SI, Dehghani F, Korf HW, Hailer NP.

Sprouting of corticospinal axons in co-cultures of rat motorcortex and spinal cord.

2008, submitted.

Frankfurt am Main, den

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Stavros Stavridis