Axonal regeneration in hippocampal and spinal cord organotypic slice cultures

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LIST OF ABBREVIATIONS:

Ab	antibody
BDA	biotin dextran amine
cAMP	cyclic AMP
CNS	central nervous system
CSPG	chondrotin sulphate proteoglycan
CST	corticospinal tract
DG	dentate gyrus
DIV	days in vitro
DRG	dorsal root ganglion
EC	enterohinal cortex
GAG	glycosaminoglycan
GFX	GF109203X
Gö	Gö6976
GPI	glycosylphosphatidylinositol
MAG	myelin associated glycoprotein
MBP	myelin basic protein
MOG	myelin oligodendrocyte protein
LY	LY294002
NgR	Nogo Receptor
NGS	normal goat serum
OMgp	oligodendrocyte-myelin glycoprotein
OEC	olfactory ensheathing cells
P0, P6	postnatal day 0, postnatal day 6
PBS	phosphate buffered saline

PFA	paraformaldehyde
PI3-K	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PNS	peripheral nervous system
PP	perforant path
PTX	pertussis toxin
RGC	retinal ganglionic cells
ROCK	Rho kinase
RT	room temperature
SC	spinal cord
SCI	spinal cord injury
TBI	traumatic brain injury
VAChT	vesicular acetylcholine transporter
XSp	xestospongin

SUMMARY:

Under normal conditions, axonal regeneration after lesions is not possible in mature CNS but can occur in embryonic and early postnatal nervous systems. In recent years, a number of possible strategies to enhance axonal regeneration and eventually treat spinal cord and brain injuries have been identified, some of which have been used successfully in animal experiments, but till now there is still no successful treatment available for patients. This problem is partly due to the complexity of the animal experiments which makes it difficult to compare different treatment strategies.

In this project, we have used organotypic slice culture models to test the effectiveness of pharmacological compounds that interfere with various signal transduction mechanisms, to promote axonal regeneration. We used the entorhino-hippocampal slice cultures to assess regeneration of entorhinal fibers projecting to the dentate gyrus after mechanical lesions and treatment. It was previously shown (Prang *et. al.*, 2001) that there is a marked decrease in regenerating fibers when a lesion is made at 6-7 days in vitro or later in slices derived from postnatal day 5-6 mice. We took this as a control model where there is little spontaneous axonal regeneration, added treatments on the day of lesion and later traced for entorhinal axons with biotinylated dextran amine (BDA). In this study it was shown that compounds acting on the cAMP, PKC and G-proteins can promote regeneration. Furthermore, we have identified the inhibition of the PI3-kinase pathway and the IP-3 receptor as potential drug targets that promote axonal regeneration.

In order to study axonal growth in a spinal cord environment we have developed a spinal cord longitudinal organotypic slice culture model which allowed us to follow axons along the rostro-caudal extension of the spinal cord. Slices of cervical spinal cord were cut in the sagittal plane from early postnatal mice and were maintained in culture for various time periods up to 4 weeks. Histological and immunohistochemical stainings of the cultures have shown that these slice cultures maintain the ventro-dorsal polarity of the spinal cord and that an intrinsic fibre projection develops which runs along the rostro-caudal extension of the spinal cord slice culture. After mechanical lesion, these fibres have the ability to regenerate spontaneously demonstrating the intrinsic ability of the spinal cord for repair, but this ability is decreased with increasing time in culture. During the culture period the axons became myelinated and

expressed synaptic markers. These cultures could thus serve also as a model for myelin formation and synaptogenesis.

We have analyzed the potential of axons from longitudinal spinal cord cultures to grow into an adjacent slice of cerebellar tissue. We could show that spinal cord axons do enter the cerebellar slice in particular when early postnatal spinal cord is combined with postnatal cerebellum. Pharmacological treatments were used to enhance axonal growth. Similarly to our findings in the entorhino-hippocampal model, cAMP activators and PKC inhibitors promoted axonal growth from the spinal cord to the cerebellum. In cocultures of longitudinal spinal cord slices with cortical slices we have shown that fibers from the cortical slices grew extensively into the spinal cord slice and extended caudally for substantial distances.

Our results demonstrate that organotypic slice cultures can be a useful tool to study axonal growth and regeneration. Intrinsic spinal cord axons have a considerable potential for spontaneous regeneration in the early postnatal period and are able to grow both through a mechanical lesion and into another tissue. Moreover, compounds interfering with signal transduction mechanisms, particularly cAMP, PKC, PI3-Kinase, G-proteins and IP3 receptors, were able to promote axonal growth and regeneration in diverse slice culture models making them interesting drug candidates for the promotion of axonal regeneration.

1.   GENERAL INTRODUCTION

1.1.   Axonal degeneration in the CNS

CNS damage can occur in many ways. One major cause is neurotrauma which can occur after head injury, i.e., traumatic brain injury (TBI) or in the spinal cord, i.e., spinal cord injury (SCI). Other major causes are various neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease; neuroinflammatory diseases such as multiple sclerosis; and neurovascular diseases such as stroke. All these conditions will result in the loss of neurons and in the damage to axons leading to a severe dysfunction of the CNS. In the adult CNS, the loss of neurons normally cannot be compensated by the generation of new cells from immature precursors, and damaged axonal projections fail to regenerate (Cajal, 1928). However, axons in the embryonic and early postnatal CNS do regenerate after injury, and it is in the perinatal period that the regenerative potential of axons is lost (Martin GF, 2000). In this thesis we have used the in vitro model system of organotypic slice cultures of different CNS regions in order to characterize this decrease of regeneration and have tried to induce axonal regeneration by interfering with various signal transduction mechanisms. Because of the extraordinary importance of spinal cord injury and the absence of a suitable in vitro system to study axonal growth and regeneration in the spinal cord we have developed a new spinal cord slice culture model.

1.1.1   The impact of traumatic injury to the CNS

There are around 1.5million people each year who sustain a TBI in USA alone. Out of these around 230 000 are hospitalized and survive, 50 000 die and around 85 000 suffer from long-term disability. The cost of TBI in US in 1985 was around USD38 billion (Thurman DJ, 1999). There are also 10 000 new SC injuries per year, and around 250 000 people living with SCI in USA. This has great impact not only on the lives of the patients and their families, but also on economy. It is estimated that the annual cost of SCI is around USD7.7billion (DeVivo, 1997).

SCI is classified by the American spinal cord injury association using the following ASIA impairment scale, as described in Fig. 1.1 (Thuret S, 2006): Scale A: complete lesion, no motor or sensory function is preserved in the sacral segments S4-S5; B: incomplete lesion, sensory but not motor function is preserved below the neurological level and includes the sacral segments S4-S5; C: incomplete lesion, motor function is preserved below the neurological level, and more than half of key muscles below the neurological level have a muscle grade less than 3, i.e. active movement with full range of motion against gravity but no resistance; D: incomplete lesion, motor function is preserved below the neurological level, and at least half of key muscles below the neurological level have a muscle grade of 3 or more; and E: motor and sensory function are normal.
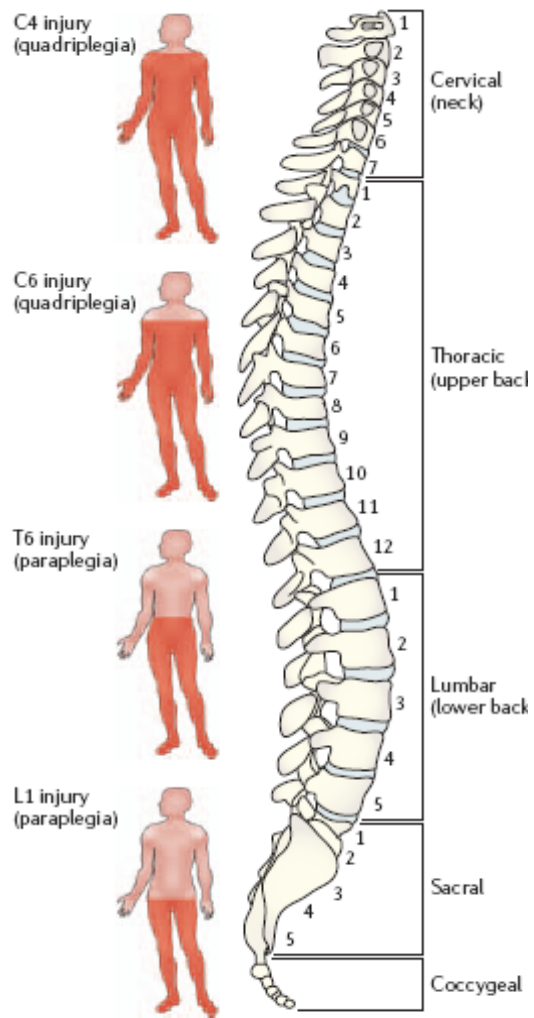
Fig. 1.1 ASIA Impairment Scale: Spinal cord injury severity as classified by the American Spinal Cord Injury Association (taken from Thuret, 2006)

1.1.2   The neurobiology of axonal regeneration

Ground-breaking work by Ramón y Cajal confirmed that a few days after injury, there is axonal sprouting at the lesion site (Cajal, 1928). Seventy years later, David and Aguayo showed that retinal ganglionic cells (RGCs) could regenerate their axons through peripheral nerve grafts (David S, 1981). Caroni and Schwab have later shown that adult CNS is nonpermissive for axonal regeneration to take place, and this is due to axon growth inhibitors associated with myelin (Caroni P, 1988a; Caroni P, 1988b).

Nowadays we know that the reduced regeneration capacity of CNS axons is due to several factors, including the activation of axonal-growth inhibition related to the lesion, the presence of inhibitors in the adult CNS and other signalling mechanisms inhibiting growth. These will be further discussed in this thesis. For regeneration to take place, many factors need to be targeted. Most important, the damaged neuron must survive, and axon keep contact or re-extend its processes and make synapses to its original neuronal targets. In addition, these contacts should also be functional, thus the axon should be remyelinated. (see Fig. 1. 2; for a review refer to Horner and Gage, 2000).
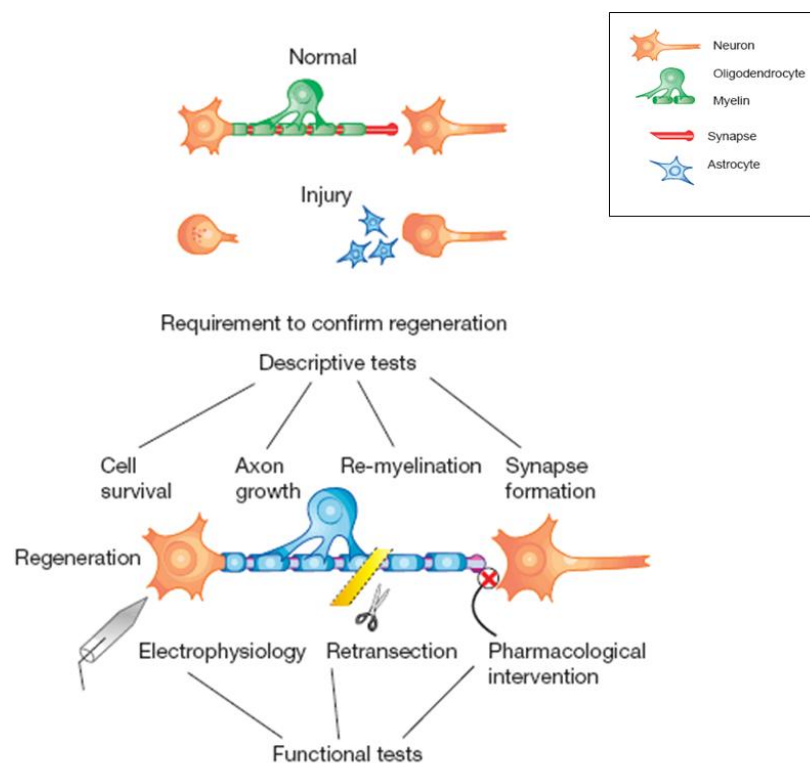
Fig. 1.2 Steps needed for axonal regeneration to take place (taken from Horner and Gage, 2000)

In recent years, various ways to improve axonal regeneration have been identified. Unfortunately, so far we still need to have better treatment for SCI and TBI. Recently, there were various promising clinical studies going on for SCI. Of particular interest are the cell permeable Rho inhibitor Cethrin developed by BioAxone on ASIA category A patients (Baptiste DC, 2006; Thuret S, 2006). Another clinical trial on ASIA A patients using anti-Nogo-A antibody, which was developed by M. Schwab and his colleagues (Schnell L, 1990; Fouad K, 2004) is taking place in Switzerland (Thuret S, 2006).

1.2   Factors involved in axon regeneration failure

There are various factors that account for axon regeneration failure in adult CNS (for a review, refer to Goldberg and Barres, 2000). These can be divided into intrinsic and extrinsic factors. Here we will have a more in-depth overview of these factors and the mechanisms by which they effect regeneration. In a later chapter (Chapter 1.3) we will then have an overview of the treatment methods to target these mechanisms and induce regenerative growth.

1.2.1   Extrinsic factors:

a.   *Glial scar*

After injury, the glial scar is one of the major obstacles for axonal regeneration to take place (Fig. 1.3, Silver J, 2004). The glial scar acts both as a physical barrier, but also inhibits axonal growth due to inhibitory molecules within the scar itself (David S, 2003).

On injury, microglia, oligodendrocyte precursors, meningeal cells and astrocytes are attracted to the lesion site. This forms a thick cellular barrier which inhibits axons from crossing through the scar. These axons were described by Ramón y Cajal as having 'dystrophic endballs' which he thought are incapable of regeneration (Cajal, 1928). Later studies show that these endings are highly active structures, which do not lose their ability to regenerate. Moreover the glial scar requires a certain time to mature and block axonal growth (Silver J, 2004); in adult rats it can take around 2 weeks (Berry M, 1983). This gives a 'window of opportunity' for regeneration to take place. The glial scar as a physical barrier also has a beneficial effect, as it can isolate the injury site and decrease the area of inflammation and cell death (Yiu G, 2006).
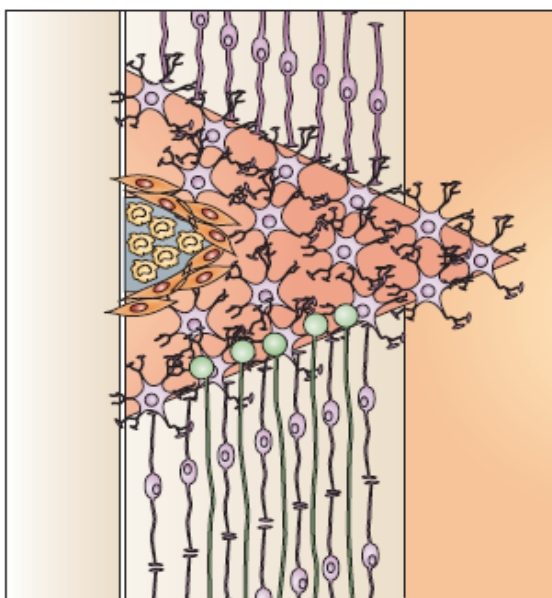
Fig. 1.3 Glial scar formation through a large stab lesion Astrocytes align at the lesion site forming a barrier which inhibits growth. Later, astrocytes produce CSPGs which also inhibit growth (taken from Silver and Miller, 2004)
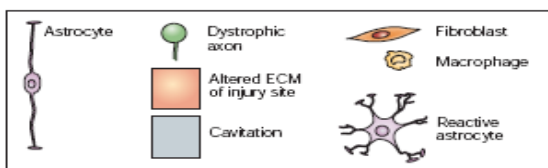
Reactive astrocytes in the glial scar are rich in proteoglycans, particularly CSPGs (including aggrecan, brevican, neurocan, versican, phosphacan and NG2) which inhibit axonal growth (McKeon RJ, 1991). CSPG secretion starts within 24h after injury and can last for several months (Jones LL, 2003). CSPGs have a growth inhibiting effect, although in some cases they can also have growth promoting effect, and during development play a role in guiding axons to their appropriate destinations (Snow DM, 1990). CSPGs are molecules characterized as large, highly sulphated glycosaminoglycan (GAG) chains attached to a protein core. The inhibitory effect of proteoglycans is due to GAG and can be neutralized with chondrotinase ABC, an enzyme that removes GAG chains from the protein core (Silver J, 2004). It is thought that the inhibitory effect of CSPGs is mediated through a signalling pathway involving RhoA (Dergham P, 2002). In addition to CSPGs, other molecules are present at the glial scar which mediate growth inhibition and are upregulated at the lesion site such as tenascines, Semaphorin3, ephrin-B2 and slit proteins (Silver J, 2004).

b. *Myelin associated inhibitors*

In addition to the effects of the glial scar, myelin associated inhibitors play a major role in hindering axonal regeneration. In fact, before the scar matures, the main hindrance for regeneration comes from inhibitor molecules associated with myelin (Filbin, 2003). These include Nogo, MAG, OMgP (Fig. 1.4), and others (such as semaphorin 4D and ephrin B3 which will not be discussed in this thesis). Around 20 years ago, Schwab and his colleagues showed that CNS myelin contains molecules which inhibit axonal growth and regeneration (Caroni P, 1988b). A monoclonal antibody against myelin protein named IN-1 (later called Nogo) was than generated which was able to at least partially neutralize the inhibitory effects of CNS myelin (Caroni P, 1988a; Schnell L, 1990). NOGO is probably the best known myelin associated inhibitor. It exists in 3 forms Nogo – A, − B and –C, which origin from a single gene and have a common C-terminal domain of 188 amino acids which 'anchors' the Nogo; a property shared by the reticulon protein family. It was independently studied by 3 different labs (Chen MS, 2000; GrandPré T, 2000; Prinjha R, 2000) using different cloning approaches; which also yielded different results related to axonal regeneration. Nogo-A is the largest, and is highly expressed on the surface of oligodendrocytes (Chen MS, 2000; GrandPré T, 2000), and on the innermost loop of myelin (Huber AB, 2002), where it can contact axons. Nogo-A contains 2 inhibitory domains − amino-Nogo (soluble and found only on Nogo-A) and Nogo-66. Nogo

receptor (NgR) a GPI anchored protein, has been cloned based on the identification of Nogo-66 (Fournier AE, 2001). NgR forms a complex with p75 (Wang KC, 2002b), p75 being one of the co-receptors for Nogo, MAG and OMgp (Wang KC, 2002a).

Myelin-associated glycoprotein (MAG) is another myelin associated neurite growth inhibitor (Mukhopadhyay G, 1994; McKerracher L, 1994). It is a member of the immunoglobulin superfamily, containing five immunoglobulin-like domains in its extracellular region. It is expressed on both CNS oligodendrocytes and PNS Schwann cells and is thought to be involved in the formation and maintenance of myelin. In culture, MAG induces axonal (but not dendritic) growth cone collapse (Shibata A, 1998). Its inhibitory effect is restricted to adult neurons, while in young neurons it has a growth-promoting effect. This transition occurs with neuronal maturation and is thought to be cAMP dependant (Cai D, 2002).
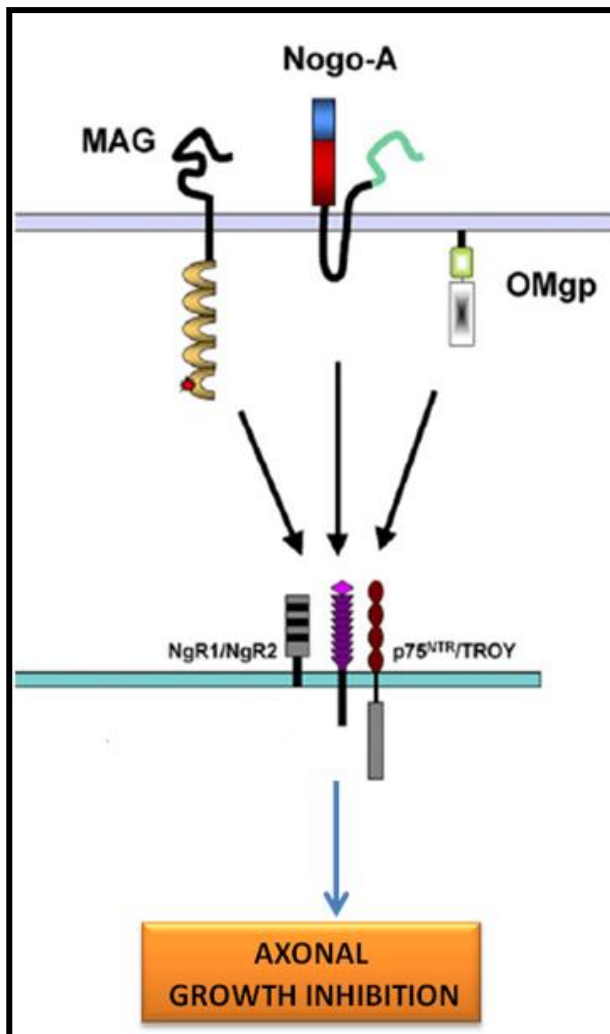
Another myelin associated inhibitor identified more recently is oligodendrocytes myelin glycoprotein OMgp (Wang KC, 2002b). OMgp is a GPI (glycosyl phosphatidylinositol) − linked protein, and is localized at the surface of myelin (Mikol DD, 1990). It is not only highly expressed in oligodendrocytes, but also in several neurons (Habib AA, 1998) and in the PNS (Mikol DD, 1990).

Fig. 1.4 Myelin Associated Inhibitors inhibit axonal regeneration through various receptors. (modified from Hannila et. al., 2007)

c. *Signalling mechanisms*

Various signalling mechanisms contribute to either the promotion or the inhibition of axonal regeneration. Pharmacological treatments targeting these mechanisms should thus help improve growth. This chapter attempts to give a general overview of such mechanisms. In Chapter 1.3.4 a-d, the relevant compounds affecting these mechanisms will be discussed in further detail.

Promoter signals

A well known promoter is cAMP (see also Fig. 1.5). Neurotrophins activate cAMP (Cai D, 1999) (by activating Trk and Erk receptors, which in turn produces a transient inhibition of PDE4 activity). In turn, cAMP activates PKA, which than activates CREB. This will allow neurons to overcome inhibitors of myelin and induce axonal growth (Hannila SS, 2008). An increase in cAMP also increases the levels of Ca^{2+} within the neuron, which activates calcium – calmodulin – dependant protein kinase II (CaMKII) and also promotes axonal growth (Wen Z, 2004). Filbin and her colleagues have done several studies which show that on increasing cAMP, axonal regeneration is promoted. Of particular interest is pharmacological activity of the cAMP promoter Rolipram, which can be a promising drug to induce axonal regeneration.
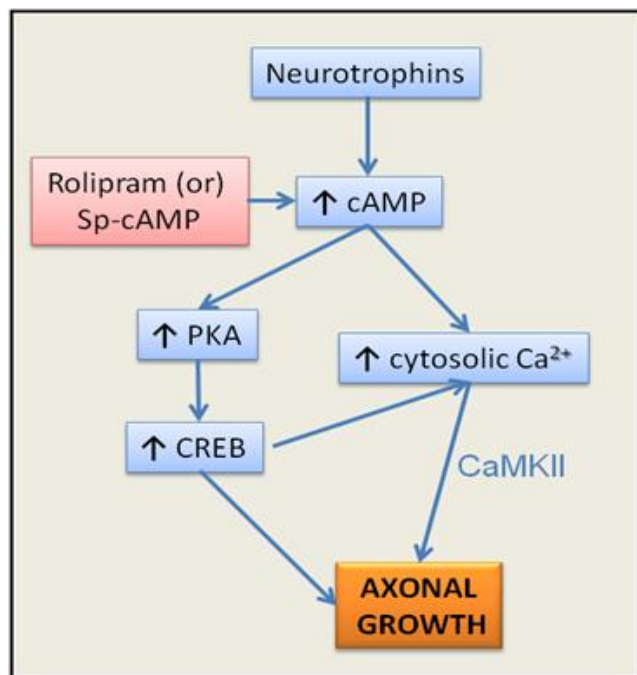
Fig. 1.5 cAMP pathway and its agonists promote axonal growth

It is well known that phosphoinositide 3-kinase (PI3-K) signalling pathway affects growth, survival, and movement of cells. Moreover, this pathway plays an important role in axonal and dendritic morphogenesis during nervous system development, particularly axonal elongation and guidance (Rodgers EE, 2002). In addition, the PI3-K signalling is essential for the maintenance of the neuronal connectivity within the adult brain (Kwon CH, 2006). There are at least 2 different pathways by which PI3-K controls axonal growth. PI3-K recruits TrkA receptors

to the axonal growth cone; this inhibits downstream Rho activation (discussed above), thus promoting axonal growth. PI3-K signalling also promotes axonal elongation by activating Akt. In turn, Akt inhibits GSK-3β, leading to increased microtubule stability. LY294002 (LY) is a specific inhibitor of PI3-K activity (Vlahos CJ, 1994). While it was shown that acute treatment of sensory growth cones with LY294002 had a collapsing effect on growth cones, these growth cones recovered rapidly and resumed outgrowth in the continued presence of LY294002. Chronic LY294002 treatment as applied in our cultures might desensitize the PI3K/Akt pathway and make neurites unresponsive to signals normally inducing inhibition of axonal growth through inhibition of the PI3K/Akt pathway.

Inhibitory Signals

The Protein Kinase C (PKC) pathway (Fig. 1.6) is known to regulate axonal growth by acting on cytoskeleton regulators like GAP-43 (Laux T, 2000). Protein Kinase C (PKC) belongs to the family of kinases, which have key roles in regulating multiple cellular activities. Activation of PKC might be involved in differentiation and initiation of neurite outgrowth in PC12 cells (Korshunova I, 2007); it is also involved in the transmission of inhibitory signals leading to growth cone collapse (Conrad S, 2007). Myelin inhibitors and CSPGs are shown to induce PKC activation. In turn, PKC activates Rho (discussed later) which is involved in signal transduction mechanisms inhibiting axonal growth (Sivasankaran R, 2004). Blocking of this pathway should lead to axonal growth. Prang *et. al.* have shown for the first time that pharmacological inhibition of PKC activity does indeed promote regenerative axonal growth. Sivasankaran *et. al.* have taken this a step further by showing that delivery of the PKC inhibitor Gö6967 into the lesion site promotes regeneration of dorsal column axons in the spinal cord in-vivo. This may also prove to be a promising treatment in the future.

Another well known intracellular signalling molecule which inhibits axonal regeneration is RhoA (Fig. 1.6). The Rho family of small GTPases is known to transduce extracellular signals to the actin cytoskeleton to modulate growth cone motility. While some of these members induce growth, RhoA inhibits growth by inducing growth cone repulsion and collapse. Various groups have demonstrated that RhoA is activated by myelin associated inhibitors (Yamashita T, 2002, Fournier AE, 2003). Myelin inhibitors activate RhoA by recruiting Rho-GDI, which interacts with p75(NTR) to allow the release of Rho-GDP from its bound state leading to the

release of Rho (Yamashita T, 2003). One of the downstream effectors of Rho is ROCK (Rho kinase). The activation of ROCK induces growth cone collapse through its multiple downstream effectors. In animal models of SCI, delivery of the ROCK antagonist, Y-27632 was shown to promote regeneration and functional recovery.
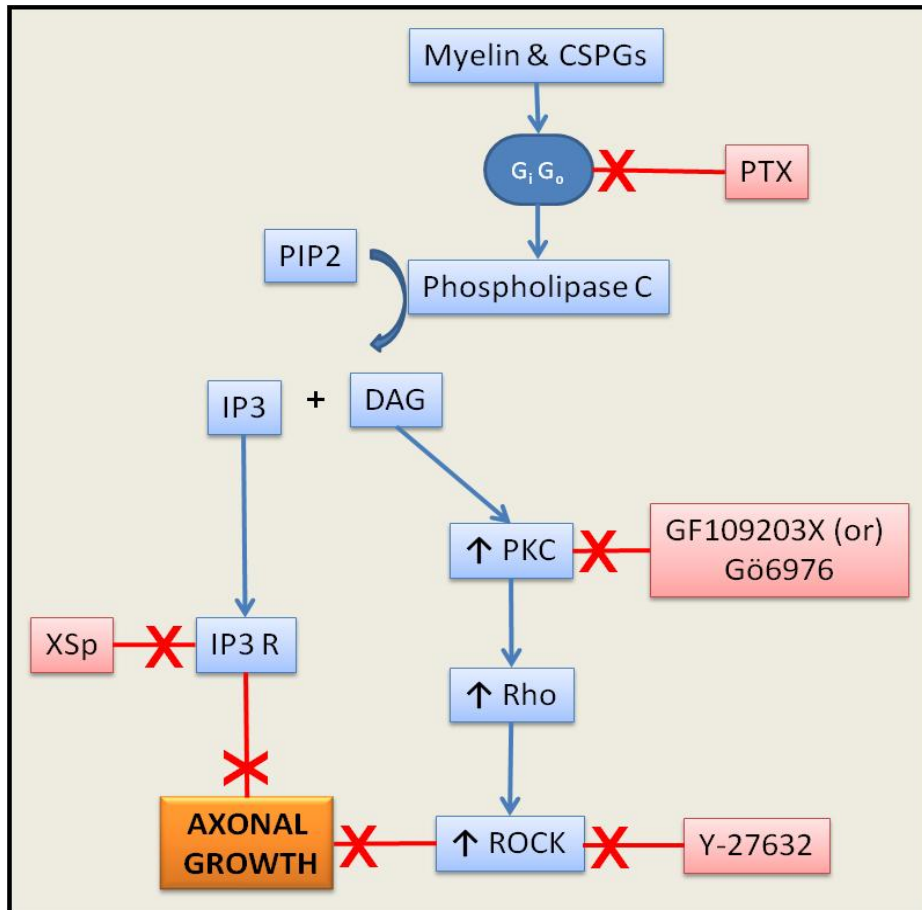
Fig. 1.6   Signals inhibiting axonal growth and their antagonist drugs

Pertussis toxin (PTX) inhibits signal transduction through the PI3K/Akt pathway (Fig. 1.6). It acts by binding to G_i- and G_o-proteins to inactivate them (Post GR, 1996). G-proteins are known to inhibit cAMP and promote PKC production; thus by inhibiting G-proteins, there is an increased cAMP level and improved axonal regeneration. It was previously shown that PTX allows neurotrophin-treated neurons to overcome inhibition by myelin (Cai D, 1999; Igarashi M, 1993). In addition, Pertussis induced extensive growth in lesioned organotypic slice cultures (Prang P, 2001). Following these results, although in-vivo studies are currently not available, PTX and other G-protein inhibitors should be further investigated as a potential treatment of CNS injury.

In addition to Protein kinase C, the IP3 signalling pathway (Fig. 1.6) is also activated by myelin inhibitors through activation of phospholipase C (Hasegawa Y, 2004) which acts on both PKC and IP3 (see Fig 1.6). Phospholipase C activation leads to the hydrolysis of PIP_2 to produce diacylglycerol (DAG) and IP_3 (Berridge, 1998). IP3 then binds to the IP3 receptor to induce release of $Ca2^+$ from the intracellular store. DAG together with an elevation of Ca^{2+} directly activates PKC to inhibit growth. Activation of the IP3 receptor thus also might be beneficial for inducing axonal regeneration in an inhibitory environment, although on the other hand IP3 receptor activation has also been implicated in the stimulation of neurite outgrowth (reviewed in Hasegawa Y, 2004). Although the growth promoting effects of Xestospongin have not yet been studied, it might promote axonal regeneration in an inhibitory environment.

1.2.2   Intrinsic growth potential of lesioned axons

Intrinsic factors are thought to influence axonal growth capability. The growth cone cytoskeleton modulators GAP43 and CAP23 (Skene JH, 1981; Aigner L, 1995), are downregulated in the CNS during development (Laux T, 2000). These proteins can induce axon elongation in adult DRG neurons in vitro, and trigger an increase in regeneration of DRG axons in adult mice after spinal cord injury in vivo (Bomze HM, 2001). The downregulation of GAP43 is inversely related to myelination. In adult rats manipulated to lack myelination in the spinal cord, GAP43 expression is strongly increased (Kapfhammer JP, 1994a; Kapfhammer JP, 1994b). Another potential intrinsic stimulator of axonal growth is the anti-apoptotic gene, Bcl-2. It was shown that Bcl-2 promotes regeneration of retinal axons in vivo (Chen DF, 1997). Unfortunately, these results could not be reproduced, and other studies show that Bcl-2 overexpression increases cell survival but there is no regeneration both in vivo (Lodovichi C, 2001; Inoue T, 2002), and in vitro (Solé M, 2004).

cAMP is also essential in regulating axonal growth. There is high expression of cAMP levels during development. Axonal growth is promoted by inhibiting PKA, a downstream effector of cAMP; thus preventing inhibition by MAG. In the adult CNS, cAMP levels are low and are inhibited by MAG (Cai D, 2002). After experimental elevation of cAMP levels (e.g. by conditioning lesion Neumann S, 1999), there is a significant increase in regeneration in adult CNS after lesion (Spencer T, 2004).

1.3   Experimental approaches to improve axon regeneration

Various approaches to promote axonal regeneration and functional recovery have been studied in many labs. Some of these studies have even been extended to use in patients in clinical trials and might one day be used to treat TBI and SCI. In this section we will get a general overview of the various treatment methods and mechanisms used to promote axonal growth.

1.3.1   Transplantation and Cell Replacement

a. Transplantation of Schwann cells and OECs

As we have discussed earlier, axonal regeneration can occur in the PNS but not in the CNS. In the 1980s, Aguayo and his colleagues used this idea to graft permissive Schwann cells in CNS tissue (Fig. 1.7) (David S, 1981; Bray, 1987). This resulted in axons growing well into the grafted PNS, but very few managed to leave the graft back to the CNS. Thus there was limited functional recovery.
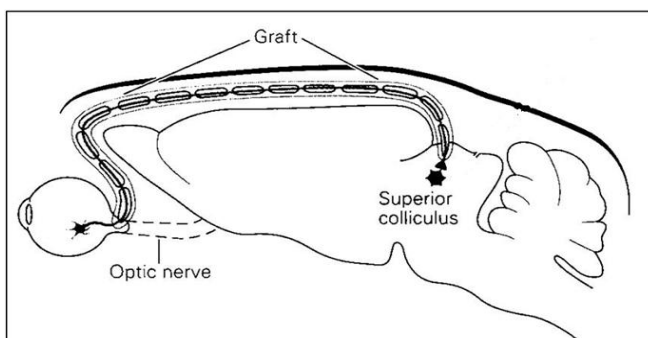
Fig. 1.7 Grafting of PNS tissue in CNS done in the 1980s by Aguayo and colleagues (adapted from Bray, *1987)*

A newer approach is the transplantation of olfactory ensheathing cells (OECs). OECs are produced from stem cells in the olfactory mucosa and can be cultured from the adult olfactory bulb or mucosa (Raisman G, 2007). Unlike Schwann cells, OECs are supposed to 'cross back' from the grafted tissue to the CNS (Li Y, 1997) and migrate over long distances within the host tissue (Ramón-Cueto A, 1998). Moreover, OEC grafts promote myleination, growth factor secretion and enhancement of sprouting, leading to functional recovery even in completely transacted SC (Ramón-Cueto et al., 2000). These optimal results using OEC transplants led to the idea that they could provide the basis of a new therapy in humans. A clinical study in spinal

cord injured patients was recently done in China. Unfortunately, there were various medical complications and no clinically significant functional recovery was observed (Dobkin BH, 2006).

b.   Transplantation of young cells

Transplantation of young permissive tissue can also promote axonal growth. In a study by Li *et. al.,* 'old' hippocampal cultures were co-cultured with 'young' EC from early postnatal rats. In these cultures there was a robust axonal projection from the EC to the hippocampus (Li D, 1995). The same group also transplanted embryonic EC in adult hippocampus in-vivo, and showed that the amount of projection made by this tissue depends on the timing of the transplant (Zhou CF, 1989). In another study, human embryonic neural tissue transplanted in rats yielded long axonal projections along the nigrostriatal and the corticospinal tract (Wictorin K, 1990). In an adult rat model of temporal lobe epilepsy, CA3 was grafted from young rats as a way to restrain mossy fiber sprouting. In these rats, a strong axonal projection formed, reducing the amount of sprouting and seizures (Shetty AK, 2005).

c.   *Transplantation of Stem Cells*

Stem cell transplantation is another alternative to help improve axonal repair. Stem cells can come from both fetal and adult tissue; but using autologous adult progenitor cells has the advantages that the cell transplants are not rejected and also reduces various ethical concerns. Adult stem cells can be obtained from various tissues, including brain, spinal cord, olfactory system, bone marrow and blood. Moreover, nowadays stem cells can be obtained from the umbilical cord and stored until need for use. Various successful stem cell studies have been performed in rodents (Schultz, 2005). Transplantation of HSC (hematopoietic stem cells) and BMSC (bone marrow stromal cells) from adult bone marrow (Koda M, 2005); and NPC (neural progenitor cells) from brain (Karimi-Abdolrezaee S, 2006), promoted various degrees of functional recovery in mice. Some years ago, BMSC were also transplanted in SCI patients in a small clinical trial (Reier, 2004). Although improvements were observed, in such a trial it was difficult to have controls and thus assess properly the outcome of the therapy.

1.3.2 Inhibition of the glial scar

As discussed earlier, CSPGs are the main inhibitors of axonal regeneration in the glial scar (Fig. 1.3). The inhibitory effect of proteoglycans is due to GAG (glycosaminoglycan) chains (Silver J, 2004). Several studies have shown that these GAG chains can be degraded by chondrotinase ABC, thus enhancing regeneration. In rat spinal cord, this treatment was shown to improve regeneration of the nigrostriatal pathway (Moon LD, 2001), and the dorsal columns and the CST, with improved functional recovery in vivo (Bradbury EJ, 2002). In a different study, this treatment was combined with Schwann cell grafts in a spinal cord injury model, and was shown to improve regeneration (Chau CH, 2004). It was also shown in vitro that another way of overcoming inhibition by CSPGs is by inactivating Rho (Dergham P, 2002).

Other studies targeted other potential growth inhibitors in the glial scar, including GFAP and EphA4. Spinal cord injured GFAP (Menet V, 2003) and EphA4 (Goldshmit Y, 2004) knockout mice show improved regeneration and functional recovery.

1.3.3 Neutralisation of inhibitory molecules in myelin

In a first attempt to neutralise myelin associated inhibitors, IN-1 monoclonal antibody was produced (Caroni P, 1988a). IN-1 Ab was found to promote long distance axonal regeneration and functional recovery (Schnell L, 1990; Bregman BS, 1995; Thallmair M, 1998). This effect was observed in various CNS lesion models. Moreover, it was noted the IN-1 Ab leads to sprouting of both lesioned and unlesioned axons (Schwegler G, 1995; Thallmair M, 1998; Z'Graggen WJ, 1998). Recently an anti-Nogo-A specific antibody was also used successfully to treat SCI in monkey; which yielded promising results, ie, axonal sprouting and functional recovery (Freund P, 2006). This brings us a step closer to this promising therapy for CNS, particularly SC injured humans.

 To study Nogo further (Fig. 1.8), independent studies from 3 different labs made different mice deficient in Nogo-A – one lacking Nogo-A and -B (Kim JE, 2004), one lacking Nogo-A (Simonen M, 2003), while in another study both knockout models were studied (Zheng B, 2003). Although all these mice were phenotypically normal, myelin growth inhibitory properties due to Nogo were expected to be reduced. Surprisingly conflicting results were

obtained. Whereas the Strittmatter group reported a strong improvement in regeneration and functional recovery in young mice (Kim JE, 2004); in the model from the Schwab group the regeneration was more modest, which could partly be explained by the up regulation of Nogo-B (Simonen M, 2003); on the other hand the models from the Tessier-Lavigne group found no significant regeneration of corticospinal tract fibers (Zheng B, 2003). It was recently shown that these variations in regeneration are in part due to differences in the genetic background of the used mouse strains (Dimou L, 2006).

Fig. 1.8 3 isoforms of NOGO. NOGO-A is found predominantly in CNS and contains 2 inhibitory domains: a 66-amino-acid domain extracellularily (green), and amino- NOGO intracellularily (taken from Goldberg and Barres, 2000)

In an attempt to address problems related to antibody delivery, vaccination against myelin inhibitors was performed and robust regeneration and functional recovery were observed after lesion in the vaccinated animals (Huang DW, 1999). Later experiments targeting Nogo-A were also performed successfully, and, more importantly, regeneration was also obtained when using the vaccine after injury (Hauben E, 2001).

Another way to promote axonal regeneration is to target the NgR. Some years ago Nogo-66 was identified (Fournier AE, 2001) as the receptor to which Nogo, MAG and OMgp are binding to inhibit axonal growth (Wang KC, 2002a). It was subsequently shown that the Nogo-66 receptor antagonist peptide NEP1-40 (blocks Nogo only) (GrandPre T, 2002; Li S, 2004) and NgR(310) (blocks Nogo, MAG and OMgp) promote regeneration and functional recovery (Li S, 2004). In a similar study p75, which is a co-receptor of NgR was targeted, but there was no regeneration present in this case (Song XY, 2004).

Similar to the Nogo molecules, also the myelin protein MAG has been implicated in the inhibition of axonal regeneration. Using an antibody against MAG it has been suggested that inhibition of MAG promotes axonal outgrowth and regeneration (Mukhopadhyay G, 1994). To verify the inhibitory effect of MAG on regeneration, a transgenic mouse (MAG-/-) mouse was used. After injury in vivo, no increase in regeneration was found (Bartsch U, 1995). In an in vitro study using myelin from MAG-deficient mice an increase in number and length of

regenerated fibres was found when compared to wild type mice but the presence of further important inhibitors in CNS myelin of MAG-deficient mice was acknowledged in this study (Li M, 1996). Although inhibition of MAG alone does not appear to be sufficient to induce regeneration, it might contribute significantly to the inhibitory action of CNS myelin.

1.3.4   Modification of signal transduction pathways

In this chapter we will discuss further the use of some of the compounds involved in signal transduction and their effect on axonal regeneration. The mechanism of action for these treatments is found in Chapter 1.2.1c. In this project many of these compounds will be used in an attempt to improve axonal regeneration in organotypic slice cultures.

a.   PKC inhibitors

PKC is activated by inhibitors present in myelin and the glial scar, and inhibits axonal growth. Moreover, PKC activates its downstream effector Rho, which in turn inhibits growth (Sivasankaran R, 2004) (for mechanism and antagonist drug action refer to Fig. 1.6). Thus blocking PKC activity pharmacologically or genetically, should help improve axonal regeneration. Various experiments have been preformed, mostly showing that blocking PKC induces axonal growth. It was shown that myelin associated inhibitors and CSPGs could inhibit neurite extension by inhibiting PKC (Sivasankaran R, 2004), (Hasegawa Y, 2004). In the early 1990s, the PKC inhibitors Gö6076 (Martiny-Baron G, 1993) and GF109203X (Toullec D, 1991), (Martiny-Baron G, 1993) were shown to be selective inhibitors of PKC. While Gö6076 acts specifically on the Ca2+-dependent classical isoforms of PKC, GF109203X was shown to inhibit most isoforms of PKC (Martiny-Baron G, 1993). GF109203X was shown to stimulate axonal growth in hippocampal organotypic slice cultures (Prang P, 2001). In another attempt to study other PKC inhibitors (chelerythrine and staurosporine) in organotypic slice cultures it was found that regeneration of Schaffer collaterals is inhibited by staurosporine (by a mechanism though to be independent from PKC), but not by the more specific PKC inhibitor chelerythrine (Toni N, 1997).

On the other hand, an in-vivo study in rats shows that intrathecal delivery of the PKC inhibitor Gö6976 stimulates dorsal column axonal regeneration (Sivasankaran R, 2004). Although further investigations are required to show how PKC activates Rho, PKC inhibitors have shown to play an important role in the treatment of axon regeneration failure. In the future, combination therapies using drugs acting on these pathways may show to have synergistic effects.

b.   Rho & ROCK inhibitors

RhoA, together with its downstream effector ROCK, are also known to inhibit axonal regeneration by inducing growth cone collapse (Yamashita T, 2003). Pharmacological strategies to block Rho could thus promote axonal regeneration (for mechanism and antagonist drug action refer to Fig. 1.6). The enzyme C3 transferase can inactivate Rho, and treatment with this enzyme can stimulate neurite growth both in-vitro and in-vivo. In studies with spinal cord lesioned animals, delivery of C3 transferase promoted CST regeneration and improved functional recovery in mouse (Dergham P, 2002), but not in rat (Fournier AE, 2003). In another study C3 transferase treatment promoted regeneration in adult rat optic nerve after injury (Lehmann M, 1999). The main problem of C3 transferase treatment is that it is not membrane permeable, thus making it difficult to deliver to the lesion site. Another cell permeable Rho inhibitor, Cethrin is currently being investigated in a clinical trial on spinal cord injured patients (Baptiste DC, 2006).

Another way of inhibiting the effects of Rho is to target its downstream effector, ROCK. Y – 27632, an ATP competitive antagonist that blocks Rho activation was shown to promote axonal regeneration and functional recovery in spinal cord injured mice (Dergham P, 2002) and rats (Fournier AE, 2003).

c. cAMP activators

Various studies have previously shown that an increase in cAMP levels improve survival of CNS neurons in response to neurotrophic factors (Goldberg JL, 2000). As discussed previously, on elevating cAMP levels, there is a significant increase in the regenerative potential of axons in adult CNS (Spencer T, 2004) (for mechanism and antagonist drug action refer to Fig. 1.5). cAMP regulates several processes including axonal response to guidance cues, neurotrophic factors and myelin associated inhibitors (Song H, 2001). Neurotrophin treatment (BDNF and GDNF), can overcome the inhibitory effect of MAG and myelin (Song HJ, 1997; Cai D, 1999). Moreover, it is thought that neurotrophins are able to elevate neuronal cAMP levels by inhibiting phosphodiesterase 4 (PDE4), the enzyme which degrades cAMP (Gao Y, 2003; Qiu J, 2002; Neumann S, 2002). cAMP in turn leads to PKA activation which consecutively leads to activation of CREB, which in turn activates Arginase I expression, an enzyme required for polyamine synthesis (Cai D, 2002). Both PKA elevation and polyamines are thought to block the inhibitory effect of myelin (Filbin, 2003).

There are some pharmacological treatments which elevate cAMP levels, which include the cAMP analogues, sp-cAMP and db-cAMP; neurotrophins (BDNF, GDNF); Forskolin and Rolipram. The PDE4 inhibitor, Rolipram is one of the treatments used in this project. It readily crosses the blood brain barrier and its dose has an established 'optimal' therapeutic window. Rolipram was shown to promote regeneration and improve functional recovery on lesioned adult rat SC (Nikulina E, 2004). So far Rolipram was tested in patients as an antidepressant in a clinical trial, which was stopped due to the drug's side effects (nausea) (Wachtel H, 1986). Since for treating spinal cord injury, Rolipram needs only to be delivered for a shorter period of time, the benefits may outweigh the side effects and thus it might still be a promising drug for the treatment of CNS injury.

1.3.5 Summary

Table 1.1 is a summary of the various approaches used to improve axonal regeneration and functional recovery discussed above.

Target	Treatment	Results & References
Transplant and Cell Replacement		
SC	Grafts in CNS	Robust regeneration into, but not out of the graft [David, 1981; Bray, 1987]
OEC	Grafts in CNS	Robust regeneration into and out of the graft to the host tissue [Li,1997; Ramon-Cueto,1998; Ramon-Cueto,2000]
'Young' cells	1. Young EC	1. Robust axonal projection from the EC to the hippocampus in-vitro [Li,1995] and in-vivo [Zhou,1989]
	2. Young CA3	2. Strong axonal projection preventing sprouting in-vivo [Shetty,2005]
	3. Neuroblasts	3. Robust axonal projections along nigro-stratal and corticospinal tract [Wictorin,1990]
Stem cells	Various e.g. HSC, BMSC, NPC	Various degrees of functional recovery [Schultz,2005; Koda,2005; Karimi-Abdolrezaee,2006]
Glial Scar		
CSPGs	Intrathecal delivery of chondrotinase ABC	Improved regeneration of the nigrostriatal pathway[Moon,2001], dorsal column and CST, with improved functional recovery[Bradbury,2002] in rat SCI
GFAP & EphA4	Genetic deletion	Improved regeneration and functional recovery after SCI in GFAP[Menet,2003] and EphA4[Goldshmit,2004] knockout mice
Mylein-Associated Inhibitors		
MAG	Genetic deletion	No improvement in regeneration in MAG-/- mice[Bratsch,1995]
NOGO	1. Genetic manipulation	1. Results vary from regeneration & functional recovery[Kim,2004; Simonen,2003] to no regeneration[Zheng,2003]
	2. Antibody	2. Regeneration with both IN-1 Ab in mice[Thallmir,1998; Bregman,1995; Z'Graggen,1998] and anti-nogo-A specific Ab in monkeys[Freund,2006]
	3. Vaccination	3. Robust regeneration in experiments targeting myelin[Huang,1999] and Nogo-A[Hauben,2001]
NgR	Intrathecal delivery	Robust regeneration and functional recovery with bothNEP1-40[GrandPre,2002] & NgR(310)[Li,2004]
p75	Genetic deletion	No regeneration[Song,2004]
Modification of Signal Transduction Pathways		
PKC inhibitor	1. GF109203X	1. Strong regeneration in hippocampal organotypic slice cultures with GFX[Prang,2001]
	2. Gö6976	2. Dorsal column regeneration but no CST regeneration with Gö-treated SCI rats[Sivasankaran,2004]
RhoA & ROCK	1. C3 transferase	1. CST regeneration and improved functional recovery in SCI in mouse[Dergham,2002] but not in rat[Fournier,2003]
	2. Y-27632	2. CST regeneration and improved functional recovers in SCI in mouse[Dergham,2002] and rat[Fournier,2003]
cAMP activator	cAMP, Rolipram & Foskolin	Axonal regeneration and functional recovery with Rolipram [Nikulina,2004]
G-prot. inhibitor	Pertussis toxin	Regeneration in hippocampal organotypic slice cultures[Prang,2001; Igarashi,1993]

Table 1.1.   Summary of axonal approaches to improve axonal regeneration

1.4   Hippocampus

The hippocampus is located in the temporal lobe of the brain and is important for the formation of learning and memory.

1.4.1   Anatomical structure

The entorhinal-hippocampal formation (here sometimes referred to as hippocampus) has a laminar organisation and consists of the entorhinal cortex (EC), dentate gyrus (DG) and hippocampus proper (i.e. the CA1 – CA3, *cornus ammonis* regions). The EC is a major relay providing a wide range of cortical inputs to the hippocampus and transferring the information processed in the hippocampus back to the cortex. The EC is divided in clearly distinguished layers (layers I to layer VI). The cells that give rise to the perforant pathway are mainly present in layers II and III. In the DG there is continuous neurogenesis going on even in the adult; it also plays a role in the formation of new memories. The DG is also subdivided in layers, mainly the stratum moleculare and the stratum granulare. The axons of DG granule cells, called mossy fibres, extend their axons to the CA3 pyramidal cells. The subiculum is located in the transition area between the entorhinal cortex and the hippocampus; and is the region where axons are exiting the hippocampus to the entorhinal cortex.

The hippocampus proper is subdivided into CA1 – CA3 regions and has a laminar organisation which is preserved in the various regions. The principal cells are pyramidal cells (forming the pyramidal cell layer), which are interconnected by interneurons. The innermost layer is the stratum oriens which contains the basal dendrites of the pyramidal neurons, whose cell bodies are found in the stratum pyramidale. Next in the CA1 is the stratum radiatum and a more superficial stratum lacunosum-moleculare. The mossy fiber projection from the dentate gyrus which can be found in the stratum radiatum, and synapse in the stratum lucidum end in the CA3 (Amaral DG, 1989).

1.4.2 'The hippocampal circuits'

The hippocampus forms a uni-directional network, and traditionally it is described as a `trisynaptic circuit` (Anderson P, 1971; Fig. 1.9). Basically, input starting from the EC forms a projection to the DG ("perforant path", synapse 1), which is followed by a projection to the CA3 pyramidal cells ("mossy fiber projection", synapse 2), with the third projection from the CA3 to the CA1 pyramidal cells ("Schaffer collaterals", synapse 3).
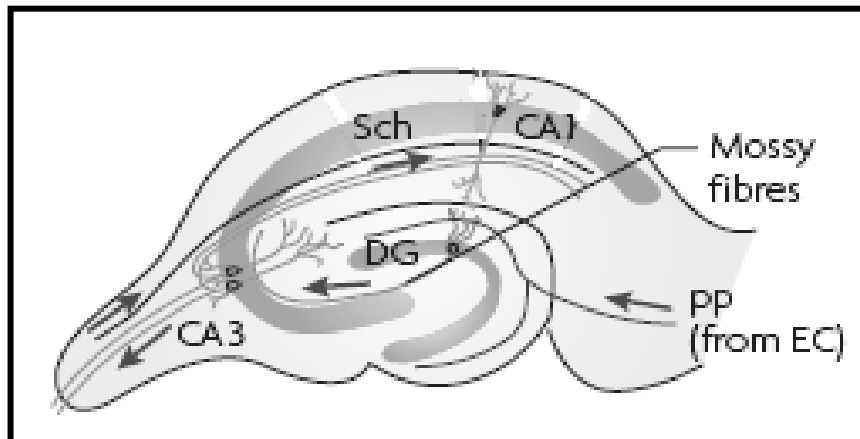
Fig. 1.9 The hippocampal trisynaptic circuit: input from the EC connects with DG in the PP, DG to CA3 as the mossy fiber projection and CA3 to CA1 as the Shaffer collaterals (modified from Neves *et. al.*, 2008)

A main characteristic of the hippocampus is its laminated organization, in which different afferents innervate different layers in a highly organized fashion. The perforant pathway originates from layers II and III of the entorhinal cortex (Amaral, 1993); with pyramidal and stellate cells (from layer II) projecting axons mainly to the DG, while small pyramidal neurons (from layer III) project their axons to the CA1 of the hippocampus. The perforant path fibers terminate in the outer molecular layer of the hippocampus, while the inner molecular layer is occupied by terminals from commissural axons. The mossy fiber pathway extends from the DG to the CA3 pyramidal cells. Multiple granule cells can synapse onto a single CA3 pyramidal cell. The Schaffer collateral pathway is derived from axons that project from the CA3 to the CA1 region. Ipsilateral axons come from CA3 neurons in the same hippocampus, while contralateral axons (better known as commissural fibres) come from the CA3 of the opposite hemisphere. Axons leave the hippocampus from the CA1 through the subiculum and back to the entorhinal cortex.

1.5   Spinal cord

The spinal cord provides a 'relay' for neurons from brain to the periphery and back.

The spinal cord is located inside the vertebral canal, and runs from the foramen magnum to the first and second lumbar vertebrae. It is covered by the spinal meninges, which further supports and protects the SC within. The SC is made of 31 segments: 8 cervical (C1 – C8), 12 thoracic (T1 – T12), 5 lumbar (L1 – L5), 5 sacral (S1 – S5), and 1 coccygeal. In the cervical part: C1 participates in the innervation of neck muscles, C2 carries sensation from the back of the head and scalp and motor innervation to muscles in the neck, C3-C5 innervate the diaphragm, and C5-T1 provide motor control for the upper extremities and related muscles. The spinal nerves enter and exit at each segment. Sensory nerves which contain their cell bodies in the dorsal root ganglia carry information to the SC via the dorsal roots. Each dorsal root contains the input from all the structures within the distribution of its corresponding body segment. Dermatomal maps portray sensory distributions for each level. These maps differ somewhat according to the methods used in their construction. Motor information is carried away from the SC via the ventral roots. The cell bodies originate from the grey matter of the ventral spinal cord.

In a cross-section of the spinal cord the area is divided in the so-called white matter and grey matter (Fig 1.10). The grey matter and is called so because it contains cell bodies (thus making it look darker). It can be further subdivided into 10 layers of Rexed (Rexed I – X); layers I – VI constitute the dorsal horn (sensory part), layer VII is the intermediate zone, VIII and IX constitute the ventral horn, and layer X surrounds the central canal. The white matter is on the external part of the SC and is subdivided into 3 columns: the dorsal column that relay somatosensory information to the medulla and in the mouse containing the corticospinal tract; the lateral column containing axons from sensory, motor and autonomic control; the ventral column containing mostly axons descending from the brain. The white matter contains the descending and ascending tracts.
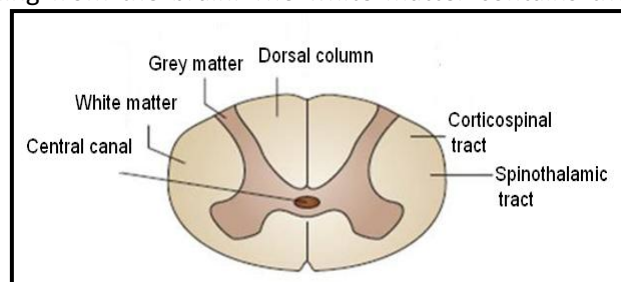
Fig. 1.10 Transverse section of the spinal cord showing grey and white matter (modified from Thuret, 2006)

Ascending and Descending tracts
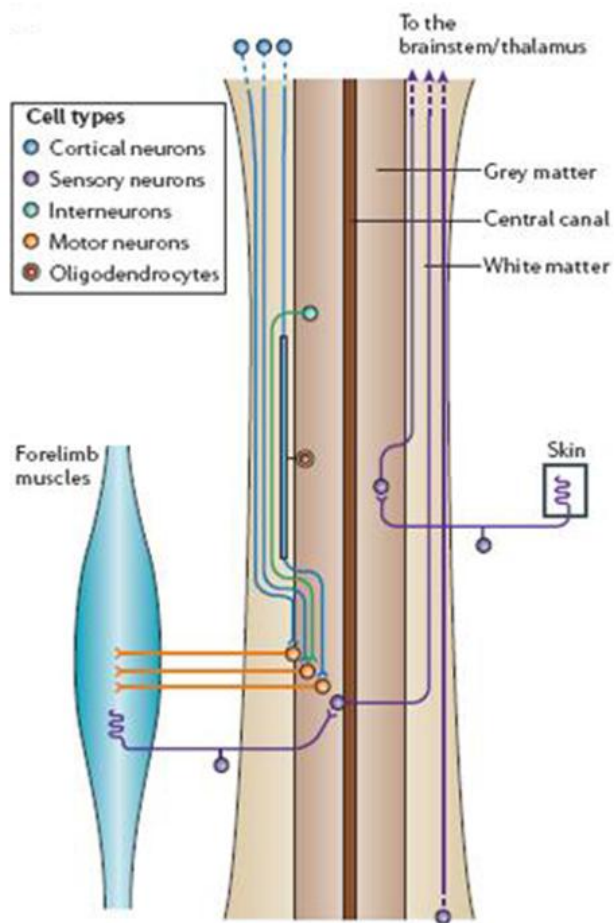
Fig. 1.11 Spinal cord: Neurons involved in ascending and descending pathways (taken from Thuret *et. al.*, 2006)

1.5.1 Major Descending tracts

The main descending tract is the corticospinal tract (CST), which mainly originates in the primary motor cortex. CST axons collect to form a lump in the medulla (called pyramid). In the medulla most axons cross (decussate) and collect in the lateral column of the spinal cord (in the human) and in the dorsal columns (in the rodent). Axons from the lateral CST synapse with alpha motor neurons and interneurons mainly from Rexed laminae IV, V, VI, and VII. The CST decreases in size at the more caudal spinal cord levels, where the fibers reach the dorsal surface of the spinal cord. CST is required for the control of movements.

1.5.2 Ascending tracts

a. Dorsal column

The dorsal column consists of sensory axons ascending to the brain. The dorsal column fibers have a faster velocity and accuracy than those of the spinothalamic tracts and are responsible for proprioception and sensation of light touch. The dorsal column fibers originate from cell bodies which are agglomerated in the dorsal root ganglion and have a peripheral branch innervating receptors located in the skin and a central branch entering the spinal cord. The dorsal columns can be further subdivided: the *fasciculus gracilis* contains fibers from sacral, lumbar, and the lower six thoracic segments and is responsible for leg sensation; while the *fasciculus cuneatus* contains fibers from the cervical to T6 of the SC and is responsible for arm sensation. The axons from the dorsal column terminate in the dorsal column nuclei in the medulla oblongata. From the medulla, neurons form an ascending bundle which synapses in the thalamus, and from there the projection continues to end in the primary somatosensory cortex located in the postcentral gyrus.

b. Spinothalamic tract

The conduction velocity in the spinothalamic tract is slower and the projection less accurate compared to the dorsal columns, this tract transmits crude types of sensations to the brain. The spinothalamic tract consists of two parts: the lateral part concerned with pain and temperature sensations and the ventral part concerned with pressure and strong touch. The lateral spinothalamic tract carries information from pain and temperature receptors in the skin, the ventral spinothalamic tract from receptors of pressure and strong touch. The sensory axons enter the spinal cord through the dorsal root and end in the dorsal horn of the gray matter. Each axon bifurcates into ascending and descending branches, which extend for $1-2$ segments and then enter the grey matter of the dorsal horn to terminate in Rexed laminae I – VI. The axons from these cells cross the midline and ascend on the controlateral side of the SC to project via the ventral posterior nucleus of the thalamus, to the somatosensory cerebral cortex.

c.   Spinocerebellar tract

The spinocerebellar tract provides information about the position of the body related to its surroundings; i.e. proprioception. It carries information from peripheral sensory receptors (in muscles and joints) which send fast conducting myelinated axons to the Clarke's column, a cell column in the lower thoracic spinal cord grey matter. From there fibers cross the midline and run to the cerebellum as the dorsal spinocerebellar tract. Additional spinocerebellar tracts are the ventral spinocerebellar tract, the rostral spinocerebellar tract and the spinocuneocerebellar tract.

1.5.3   Propriospinal connections in the SC

Propriospinal neurons originate and terminate in the SC and participate in information exchange among the spinal segments. Propriospinal neurons are present in considerable numbers in the SC, and are mostly myelinated, thus having slightly larger axons than other neurons present in the SC (Chung K, 1983). Their connections ascend and descend the SC and project to various sites including interneurons and motor neurons. These neurons are required for several behavioural and physiological responses, including the control of movement. The axon length of propriospinal neurons can vary: some are short ascend and descend only one or two segments and some long extending over several spinal cord segments. The long propriospinal neurons have axons that ascend and descend in the anterior fasciculus proprius to all levels of the SC. These neurons have a bilateral influence on the more medial motor neurons and coordinate movement of the neck and pelvis. The axons of the intermediate propriospinal neurons extend for shorter distances in the ventral part of the lateral fasciculus proprius and influence the motor neurons that innervate the more proximal muscles of the limbs. Axons from the short propriospinal neurons are only found in the cervical and lumbosacral segments and travel in the lateral fasciculus proprius. These neurons influence motor neurons that innervate the more distal muscles of the limbs.

1.6   Organotypic slice cultures

Slice cultures have been extensively used in neurobiology research particularly for studies involving growth and regeneration, but also for electrophysiology studies, synapse formation, and studies on several receptors. Various models are available, including hippocampal, cerebellar and spinal cord models.

There are different ways of preparing organotypic slice cultures (as explained below and in Fig. 1.12). The choice of technique depends on the final thickness of the slices and the survival time in culture (as reviewed by Gahwiler et al., 1997). Basically CNS tissue is dissected and sliced into 100 – 400µm sections. The slices are washed to remove any debris, and then incubated in an appropriate growth medium at 37°C with sufficient oxygenation. Sterile conditions need to be kept throughout the process.
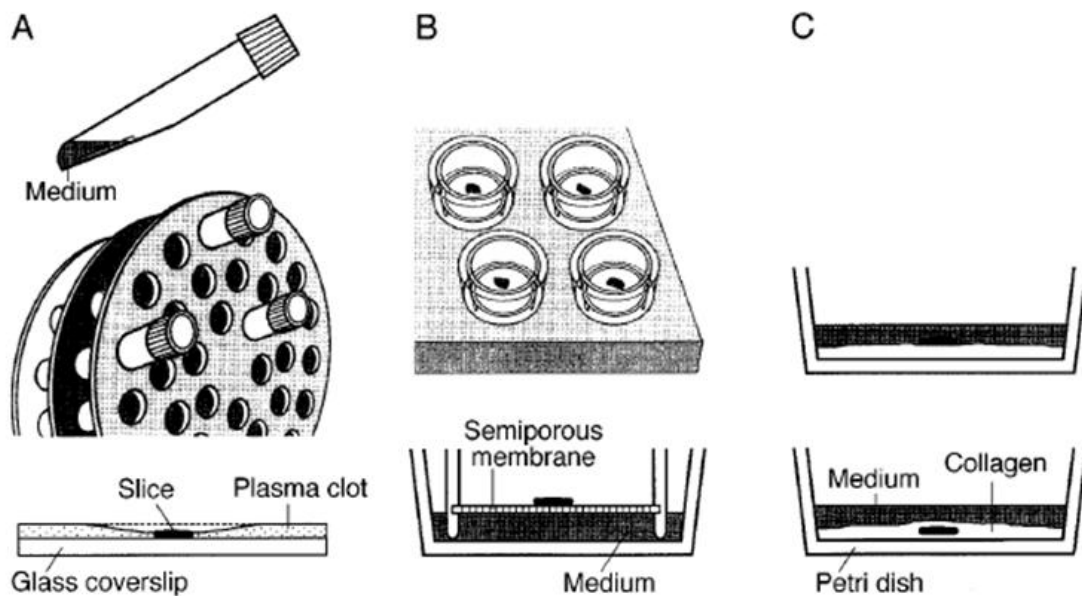
Fig. 1.12   Different techniques used for the preparation of organotypic slice cultures
A. Roller tube method, B. Membrane method and C. Culture Dish Method
(taken from Gähwiler, 1987)

In roller tube cultures, the tissue is embedded in a fibrin clot on a coverslip and then placed in a flat-sided plastic culture tube containing a small amount of medium. The tube is rotated slowly (10revs/h) to ensure continuous alteration of feeing and aeration. These cultures can survive for several weeks and are supposed to become thinner than the corresponding static membrane cultures (Fig. 1.12A; Gähwiler, 1987). In membrane cultures, slices are placed on a

semi-porous membrane (millicell or Transwell), with medium added at the bottom of the membrane, while oxygen is accessed from the surface of the culture, i.e., the cultures are stationary at an air-medium interface (Stoppini L, 1991). These cultures can also survive for several weeks. This was the culture method of choice for this project (Fig. 1.12B; Gähwiler, 1987). Slices cultured in petri dishes are placed either directly on collagen coated or embedded in collagen gels and are totally covered by medium. These cultures only survive a few days and are mainly used for electrophysiology (Fig. 1.12C; Gähwiler, 1987).

1.6.1   Entorhino-hippocampal organotypic slice cultures

Hippocampal organotypic slice cultures have been used extensively not only to study axonal growth and regeneration, but also for electrophysiology studies, and other experiments on various receptors. The entorhino-hippocampal perforant pathway develops normally in organotypic slice cultures (Frotscher M, 1993), with a robust axonal projection from the EC to the hippocampus (Li D, 1993). This projection can be lesioned in vitro by cutting through the culture with a scalpel blade, and regeneration of the entorhinal fibers can be assessed.

To visualize fibers from the EC to the hippocampus after lesion, one can use either entorhinal cortex from animals with different genetic backgrounds, e.g. CB6 mice with M6 rat or tau-GFP mice; or axonal tracing methods. Hippocampal slices have been co-cultured with EC from M6 (Li D, 1993) and tau-GFP (Radojevic V, 2004) mice. In both cases, axons from the EC only could be followed growing into the hippocampal part. For tracing the fibers, tracers in various colours are available (e.g. biotin, BDA, DiI). Depending on the tracer used, tracing can be done both in-vitro and in-vivo (here we will focus on its use in-vitro), and can be used to trace from the cell body to the axon terminal (anterograde) or vice versa (retrograde). A tracer can be used both in the crystal form, i.e., by placing the crystal on top of the part of the culture to be traced; and in the liquid form, i.e., by using a microsyringe to inject it into the target neuronal region. Tracers (with different colours) can be combined to visualize fibers from different regions of the brain. One common tracer which is also used in this project is BDA. BDA can be used both as an anterograde and as a retrograde tracer. After fixing the tissue, BDA can be developed with avidin coupled either to DAB or to a fluorescent label to be viewed either with

light or fluorescence microscopy (Reiner A, 2000). In this project we will be using BDA in the crystal form as an anterograde tracer.

It was previously shown that there is a marked decrease in fibers growing into a hippocampal slice with increasing time in culture (Woodhams PL, 1993). When a lesion is made at 6 – 7 days in vitro or later in postnatal day 5-6 mice, there are very few regenerating fibers (Fig. 1.13; Prang P, 2001). In the second part of this project (Chapter 3), we take this as a control model where there is little spontaneous axonal regeneration, and check whether various treatments (described previously and in Chapter 1.3.4) applied on the day of lesion can induce

regeneration. A main advantage of hippocampal slice cultures is that they can be easily cultured and maintained for long periods of time, thus giving enough time for axonal regeneration to take place. In addition, biochemical and pharmacological treatments are easy to perform and control.
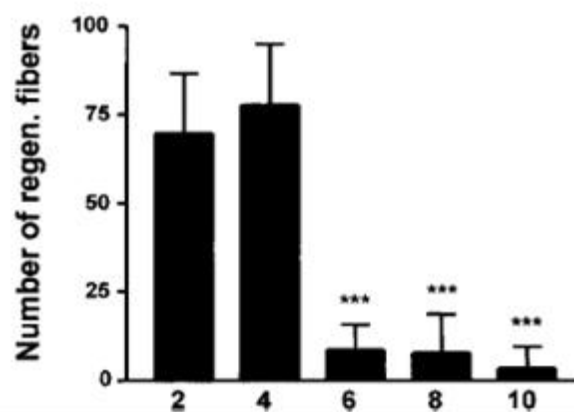
Fig. 1.13 **Histogram of regenerating fibers.** At DIV6, there is a sharp decrease in number of regenerating fibers. (taken from Prang, 2001)

1.6.2 Studies assessing axonal regeneration of the PP in organotypic slice cultures

In this project, we study the effect of compounds interfering with various signal transduction mechanisms on axonal regeneration of the perforant pathway in organotypic cultures. Similar studies using various approaches have been done before. GF109203X, a PKC inhibitor, and Pertussis, a G_i protein inhibitor, treatments have been previously studied (Prang P, 2001). There is a strong regeneration in these cultures (as was also observed in this study). In this project we extend this treatment study (as can be seen in Table 3.1).

Studies of treatment with neurotrophic factors and growth factors have been performed (Prang P, 2001), (Woodhams PL, 1996). With some of these treatments there is no significant regeneration, while GDNF and NT4 (Prang), and aFGF and Schwann cell conditioned medium (Woodhams) show a significant degree of regeneration.

The effect of ChABC (blocks the effect of CSPG) and NEP1-40 (blocks Nogo-66 binding to NgR) was also studied (Mingorance A, 2006). With treatments made immediately after lesion, both treatments promoted axonal regeneration, although the combined treatment did not have a synergistic effect. Interestingly, when NEP 1-40 treatment was delayed (at least 5 days after lesion), axonal regeneration still occurred with a similar efficiency as with immediate treatment, thus showing a promising time window when efficient treatment could still occur.

In a study by del Rio *et. al.* (del Rio JA, 2002), robust, layer specific regeneration was observed when in mature enterohinohippocampal cultures, the hippocampal part was replaced with young permissive hippocampus enriched in Cajal-Retzius cells, while the mature EC stayed in culture. Similar to what is observed in the developing hippocampus, Cajal-Retzius cells guide enterohinal axons to their target layers, i.e., there is formation of synaptic contacts with the correct target neurons. Thus CR derived signals could be used for repair.

In another approach, Bcl-2 transgenic mice were used to prevent apoptosis and promote axonal outgrowth (Solé M, 2004). In these cultures, there was no improvement in regeneration, although cell survival is improved.

Much more work could be done with this model e.g. combinations of treatments / grafting / transplantations.

1.6.3 Spinal cord organotypic slice cultures

Transverse spinal cord organotypic slice cultures have been very much studied both morphologically (Delfs J, 1989) and electrophysiologically (Rosato-Siri MD, 2004). In these cultures the intersegmental connections of the spinal cord are lost. Development and distribution of various spinal cord neurons, including somatic and autonomic motoneurons (Barber RP, 1993) and interneurons (Phelps PE, 1996) has been extensively studied.

A transverse organotypic slice culture of spinal cord co-cultured with DRG and skeletal muscle was developed to be used for electrophysiological recordings (Braschler UF, 1989). Later, the same lab used this model to study the structure and function of central synapses (Spenger C, 1991), and the electrical properties of motoneurons, muscle fibres and dorsal root ganglion

neurons (Streit J, 1991). In another study, electrophysiological measurements on transverse sections of organotypic slices were made to measure excitability of the spinal dorsal horn, which should reflect chronic pain (Lu VB, 2006). The molecular mechanism for pathfinding and synapse formation of corticospinal fibers was followed using transverse SC co-cultured with cortex (Takuma H, 2002). Longitudinal spinal cord cultures were used before in a spinal-cord cortical co-culture system (Kamei N, 2004), but the morphological properties of these cultures and the regenerative ability of the axons within were not known.

1.6.4   Organotypic co-culture systems

In a later part of this project, different co-culture models to study axonal growth in spinal cord environment will be introduced. Various organotypic co-culture models have been used to assess axonal growth and repair in neuroscience research. In entorhino-hippocampal co-cultures EC from young rat has been shown to grow into hippocampus from old mouse (Li D, 1995). We have used this culture model for the evaluation of pharmacological modulation of signal transduction pathways in order to stimulate axonal regrowth (Chapter 3). In a co-culture model of the auditory system, the medial nucleus of the trapezoid body was shown to project to the lateral superior olive (Lohmann C, 1999). Co-cultures of cortical and thalamic slices have been important for studying mechanisms of thalamocortical projections (Bolz J, 1992). The connections related to the reflex arc were also studied using a spinal cord – dorsal root ganglion – skeletal muscle co-culture system (Spenger C, 1991); while the innervations from the dorsal root ganglion to the dorsal horn was followed using foetal DRGs co-cultures with spinal cord explants (Smalheiser NR, 1981). As a general finding it is remarkable that in all of these very different preparations axonal projections maintain a high degree of order and specificity which makes such co-culture models very suitable for the study of axonal projections.

2. AIMS OF THE PROJECT

The main aims of this project were to establish new and improve currently available organotypic slice culture models to facilitate the testing of various pharmacological compounds which are thought to improve axonal regeneration.

2.1 Development of a novel spinal cord organotypic slice culture model to study spinal cord injury

In vitro models to study axonal processes and regeneration have significant advantages: they could be used to test various approaches for repair of spinal cord injury, without initially needing to use animal studies.

Organotypic slice cultures have previously been used to assess axonal regeneration in entorhino-hippocampal and cerebellar cultures. Transverse slice cultures of spinal cord were not suited for this type of study. While the cytoarchitectonic organization of the spinal cord is best preserved in these cultures, the fiber projections extending in the longitudinal direction are lost. In the first part of the project, a novel spinal cord longitudinal organotypic slice culture model was developed allowing to follow axons along the rostro-caudal extension of the spinal cord. Slices of cervical spinal cord were cut in the sagittal plane from early postnatal mice and the properties of such cultures were thoroughly studied.

The morphological properties of these cultures were studied and compared with the in-vivo situation. An important aspect was whether these cultures would really maintain the ventro-dorsal polarity of the spinal cord.

2.2 Evaluation of spontaneous regeneration of spinal cord fibres after a mechanical lesion

In this part of the project, we investigated the ability of intrinsic spinal cord axons to regenerate across a lesion. We took the culture described above (2.1) and introduced a mechanical lesion by a transverse cut through the culture with a scalpel blade.

In addition, we asked whether there would be a difference in growth across a lesion with increasing age and increasing days in vitro. It was previously observed that in the hippocampus, with increasing time in culture or with the use of slices from older postnatal

animals the regenerative potential of the axons decreased (Prang et al.). Eventually we did a proof of principle study investigating whether regeneration of intrinsic spinal cord fibers could be stimulated by a pharmacological treatment.

2.3 Evaluation of the effectiveness of treatments which promote axonal regeneration in hippocampal organotypic slice culture model

Axonal regeneration after lesion is usually not possible in the adult central nervous system but can occur in the embryonic and early postnatal nervous system. Here we used conditions where there is very little regeneration in the cultures, and tested compounds for their effectiveness to increase fibre growth across a lesion.

In this study we used the model system of mouse entorhino-hippocampal slice cultures to assess regeneration of entorhinal fibers projecting to the dentate gyrus after mechanical lesions and treatment with pharmacological compounds in vitro. This model has been used by others to study both axonal regeneration (using various methods discussed in the introduction) and functional recovery (using electrophysiology). Previous results from our lab (Prang et al. 2001) show that in entorhino-hippocampal cultures there is a marked decrease in regenerating fibers when a lesion is made at 6-7 days in vitro or later in postnatal day 5-6 mice. This DIV will be taken as a control model where there is little spontaneous axonal regeneration, in order to be able to assess the potential of various treatments to promote regeneration. By using a score system, the amount of axonal regeneration both in the treated cultures and the untreated controls was quantified. We have used compounds acting as modulators of signal transduction pathways, mainly cAMP activators and PKC inhibitors, but also other compounds acting on the IP3 and Rho pathways. We have only used 1 compound per culture, ie, no combination treatments.

3.   HIPPOCAMPUS

Modulators of signal transduction pathways can promote axonal regeneration in entorhino-hippocampal slice cultures

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The following section is based on the work soon to be submitted. Some text and figure legends might be modified from the final published version. Table 3.1 will not be part of the publication.

3.1   Abstract

Axonal regeneration after lesion is usually not possible in the adult central nervous system but can occur in the embryonic and young postnatal nervous system. In this study we used the model system of mouse entorhino-hippocampal slice cultures to assess the potential of pharmacological treatments with compounds targeting signal transduction pathways to promote re-growth of entorhinal fibers after mechanical lesions. Targeting the cAMP-system, PKC and G-proteins has been shown before to be able to promote regeneration and compounds modulating these pathways were confirmed to be active in this study. In addition we have identified the inhibition of the PI3-kinase pathway and of the IP-3 receptor as potential novel drug targets for promoting fiber regrowth after CNS lesions.

Our findings demonstrate that slice culture models can be used to evaluate compounds for their potential to promote axonal regeneration and that the pharmacological modulation of signal transduction pathways is a promising approach for promoting axonal repair.

3.2   Introduction

Axonal regeneration after lesions does not occur in the mature central nervous system (CNS). In recent years several molecules involved in the control of axonal growth in the CNS were identified, and a number of possible strategies to enhance axonal regeneration and eventually treat spinal cord injury have been proposed (for reviews see (Maier IC, 2006; Liu RY, 2006; Zhou FQ, 2006; Busch SA, 2007; Crespo D, 2007). A substantial problem in research concerned with axonal repair is the use of a wide variety of lesion models and the complexity associated with the respective *in vivo* lesion paradigms (Steward O, 2003). Unfortunately, cell and tissue culture studies are only of limited value for the study of axonal regeneration in the CNS. The important role of molecules and factors residing in the microenvironment of the lesioned fibres can only be studied in intact tissue, and not in dissociated cells or explants. This is different for organotypic slice cultures (Gähwiler, 1981; Stoppini L, 1991; Kapfhammer, 2005) which have been previously used for the study of regeneration *in vitro*. Within these cultures the cytoarchitectonic tissue organization is well preserved, and the slices can be maintained in culture for several weeks. In entorhino-hippocampal cultures, axonal regeneration was found to be abundant when the lesion is made up to 4 days in vitro (DIV), but is greatly reduced after lesions made at DIV 6 or later (Prang P, 2001). Among the different treatment strategies, the

use of pharmacological compounds aimed at modulating signal transduction pathways is particularly attractive, because ideally this way inhibitory influences from different sources might be inactivated, and the use of pharmacological compounds is much easier to apply compared to treatments with large molecules such as antibodies.

The modification of signal transduction pathways has also been used successfully in *in-vivo* models e.g. by increasing levels of cyclic AMP through inhibition of phosphodiesterase with the compound rolipram (Nikulina E, 2004), or by inhibiting the activity of protein kinase C (Sivasankaran R, 2004). In this study we have focused on the modification of signal transduction pathways after mechanical lesions in entorhino-hippocampal slice cultures. This system is well studied, and we have shown previously that there is a marked decrease in fiber regeneration when a lesion is made at 6-7 days in vitro or later in cultures derived from postnatal day 5-6 mice (Prang P, 2001). We took this as a control situation in which there is little spontaneous axonal regeneration. We then tested a number of compounds acting on different neuronal signal transduction mechanisms. Some of them are already known to promote axonal regeneration, while others had not yet been tested for their potential to promote axonal regeneration. Our results indicate that the pharmacological modulation of signal transduction pathways is a promising strategy to promote fiber re-growth in the central nervous system.

3.3   Materials and Methods

3.3.1   Organotypic Slice Cultures

Entorhinohippocampal slices were prepared from CB6F1 postnatal mouse brains (P5-6) as described by *Stoppini et al.* and *Prang et al.* (Stoppini L, 1991; Prang P, 2001). Briefly, the hippocampus was dissected out from brain slices in preparation medium (MEM with 2mM glutamax; pH 7.3) at 0°C; and 350µm transverse slices were cut using a tissue chopper (McIlwain). The slices were transferred onto a membrane (Millicell-CM, Millipore). These cultures were incubated in incubation medium (50% MEM, 25% basal medium eagle, 25% heat inactivated normal horse serum, 0.65% glucose and 2mM glutamax; pH 7.3) at 37°C in humified air with 5% CO_2 for around 2 weeks. The incubation medium was changed every second day.

3.3.2 Lesion

A lesion using a scalpel blade was made to separate the enterinal cortex (EC) from the hippocampus on cultures after 5-7 days in vitro (DIV). On the day of lesion, the medium was changed and various pharmacological agents which affect various signal transduction mechanisms (Rolipram (0.5µM; Tocris), Gö6976 (0.1µM; Biosource), LY294002 (2µM; Tocris), Farnesylthiosalicylic acid (1µM; Biomol), Pertussis Toxin (1µg/ml; Sigma), Y27632 (10µM; Alexis), Xestospongin C (0.5µM; Cayman), GF109203X (1.5µM; Tocris) and Sp cAMP (10µM) were used to treat the cultures (Table 3.1). Unlesioned cultures and lesioned cultures without any treatment were used as control. 5 days after lesion, the enterohinal axons were traced anterogradely by placing biotinylated dextran amine (BDA; Molecular Probes) on the enterinal cortex (EC) and fixed after 2 days.

Treatment	Dose	Mode of Action	Score	Range of Scores
cAMP Activators				
Rolipram	0.5µM	increases cAMP levels	2.93	**0 – 2**
Sp. cAMP	10µM	membrane permeable cAMP analogue	1.46	0 – 2
PKC Inhibitors				
GF109203X (GFX)	1.5µM	more general PKC inhibitor	3.60	0 – 3
Gö6976 (Gö)	0.1µM	PKC inhibitor specific for classical PKC isoforms	2.50	0 – 2
Other Treatments				
Y27632 (Y27)	10µM	inhibitor of the Rho target ROCK	0.66	0 – 1
Xestospongin C (XSp)	0.5µM	IP_3 receptor inhibitor, antagonises IP_3 mediated Ca^{2+} release	3.20	0 – 2
Pertussis Toxin (PTX)	1µg/ml	inhibits G(i) and G(o) G-proteins	3.03	0 – 2
Farnesylthio-salicylic acid (FTSCA)	1µM	Ras inhibitor, inhibits the MAP kinase pathway	2.16	0 – 2
LY294002 (LY)	2µM	PI3 kinase inhibitor, inhibits the PI3 kinase pathway	4.45	0 – 3

Table 3.1: Signal transduction mechanisms targeted to promote axonal regeneration in cultures

3.3.3   Immunohistochemistry

Cultures were fixed with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB) for 2 h at room temperature (RT). Cultures were washed 3 times for 10 minutes with PBS, and blocked with a solution containing 0.5% Triton X-100 and 3% normal goat serum (NGS) for 20 minutes prior to the primary antibody. The primary antibodies ABC-biotin/avidin (I : 100; DAKO) and mouse anti SMI 32 (1: 1 000; Sternberger Monoclonals, Maryland) were diluted in PBS and applied either for 2 h at room temperature or overnight at 4 °C. Sections were then washed 3 times for 10 min with PBS and incubated with the secondary antibodies Streptavidin Alexa 532 (1: 500; Molecular Probes) and goat anti-mouse Alexa 488 (1: 500; Molecular Probes, Eugene Ore.) for 2 h at RT. The slices were than rinsed with PBS and counterstained with DAPI (1: 1 000; Molecular Probes). Sections were again washed twice with PBS, and mounted with Mowiol.

3.3.4   Analysis of Slices

Only the slices that satisfied our criteria were analysed. Briefly the cultures had (1) well preserved cellular organisation and no major damages from the lesion as confirmed by SMI 32 and DAPI staining; (2) good BDA tracing with fibres extending to (and in some cases beyond) the lesion site; (3) BDA placed correctly on the EC and no spillage to the hippocampus present. Cultures were analysed using an Olympus AX-70 fluorescence microscope and pictures taken using a Spot digital camera. The brightness and contrast of these pictures was adjusted using Photoshop image processing software.

3.3.5   Semiquantitative evaluation of regeneration

Amount of regenerated fibres was analysed using a score system, depending on the percentage of fibre growth across the lesion traced by BDA, as follows: *Score 3:* >100 fibres crossing through the lesion; *Score 2:* 50 – 100 fibres crossing through the lesion; *Score 1:* <50 fibres crossing through the lesion; *Score 0:* no fibres crossing through the lesion (<5 fibers crossing through the lesion).

3.3.6   Statistical Analysis

Each experiment was performed at least 3 times with minimum number of slices (*n*) 41. The results were analysed by Kruskal-Wallis test at 95% confidence limits using Graph Pad Prism software (San Diego, California).

3.4   Results

3.4.1 Evaluation of axonal growth through a mechanical lesion in entorhino-hippocampal slices in vitro.

Enterohino-hippocampal slice cultures were lesioned at DIV5-7 to separate the EC from hippocampus, with treatment starting at the same day of lesion (Fig. 3.1). The results were analysed using a simple score system with 4 scores. The minimal score of 0 would mean that no fibers crossed, whereas the maximal score of 3 means that more than 100 fibers per slice crossed the lesion site. The scores were statistically analysed with the Kruskal-Wallis test with 95% confidence limit. All treated cultures were compared to unlesioned controls (positive control) and lesioned but untreated controls (negative controls) from the same experiment.

3.4.2   Unlesioned and untreated controls

Most of the unlesioned controls show a strong axonal projection from the EC to the hippocampus, and many fibers were labelled by BDA tracing. In most cases a score of 3 was given, signifying that more than 100 fibers were crossing the lesion site to reach the hippocampus.
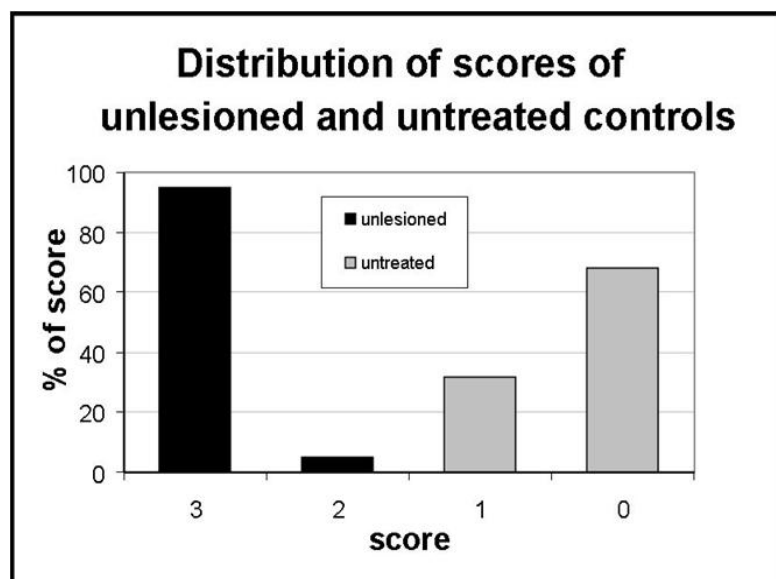

Fig. 3.1 Score distribution for unlesioned and untreated controls

In contrast, the majority of untreated controls showed either no growth or only few fibers (<50) growing through the lesion (see Fig. 3.1 for a picture showing BDA tracing for A. unlesioned and B. lesioned but untreated controls and Fig. 3.2 for score distribution for controls. The data clearly demonstrate that there is very little growth of fibers across the lesion site under control conditions.
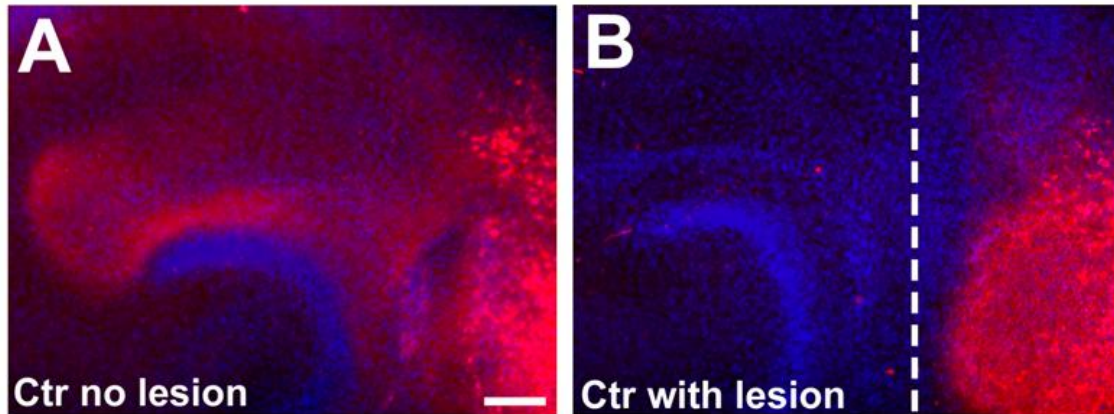
Fig. 3.2 Unlesioned and untreated controls. A. Unlesioned control having a large fibre bundle projecting from the EC to the hippocampus, traced with BDA (red); and B. Untreated control where BDA traced fibres stop at the lesion site. Scale: 100µm

3.4.3   Treatment with protein kinase C inhibitors

We have tested the protein kinase C (PKC) inhibitors Gö6976 and GF109203X (also known as bisindolylmaleimide) for their potential to promote axonal growth through the lesion site. Gö6979 is an inhibitor specific for the classical, Ca^{2+}-sensitive PKC isoforms, whereas GF109203X is a more general PKC inhibitor and assumed to be active for most known isoforms (Martiny-Baron G, 1993). In treated cultures there was a clear induction of axonal growth across the lesion (Fig. 3.3A, B) with both inhibitors. In the semiquantitative analysis there was a highly significant increase ($P<0.001$; ***) in the number of regenerating fibers in cultures treated with GF109203X (GFX, *n*= 54), and a significant increase ($P<0.05$; *) in the number of regenerating fibers in cultures treated with Gö6976 (Gö, *n*= 55, Fig. 3.4 A). Most of the cultures treated with any of the two PKC inhibitors showed growth across the lesion with >50 fibers crossing through the lesion, while some of the GFX treated cultures have >100 fibers crossing through the lesion. These results confirm and extend previous findings reported earlier from our laboratory (Prang P, 2001) and other laboratories (Hasegawa Y, 2004; Sivasankaran R, 2004) that PKC inhibitors promote axonal regeneration.

Fig. 3.3 (above) Treatments with PKC inhibitors (A and B) and cAMP Activators (C and D). The PKC inhibitors Gö6967 (A) and GF109203X (B), together with the cAMP activator Rolipram (C) show significant regeneration after treatment, while sp. cAMP (D) treated cultures do not. Scale: 100μm

Fig. 3.4 (right) Score distribution for cultures treated with (A) PKC inhibitors and (B) cAMP activators
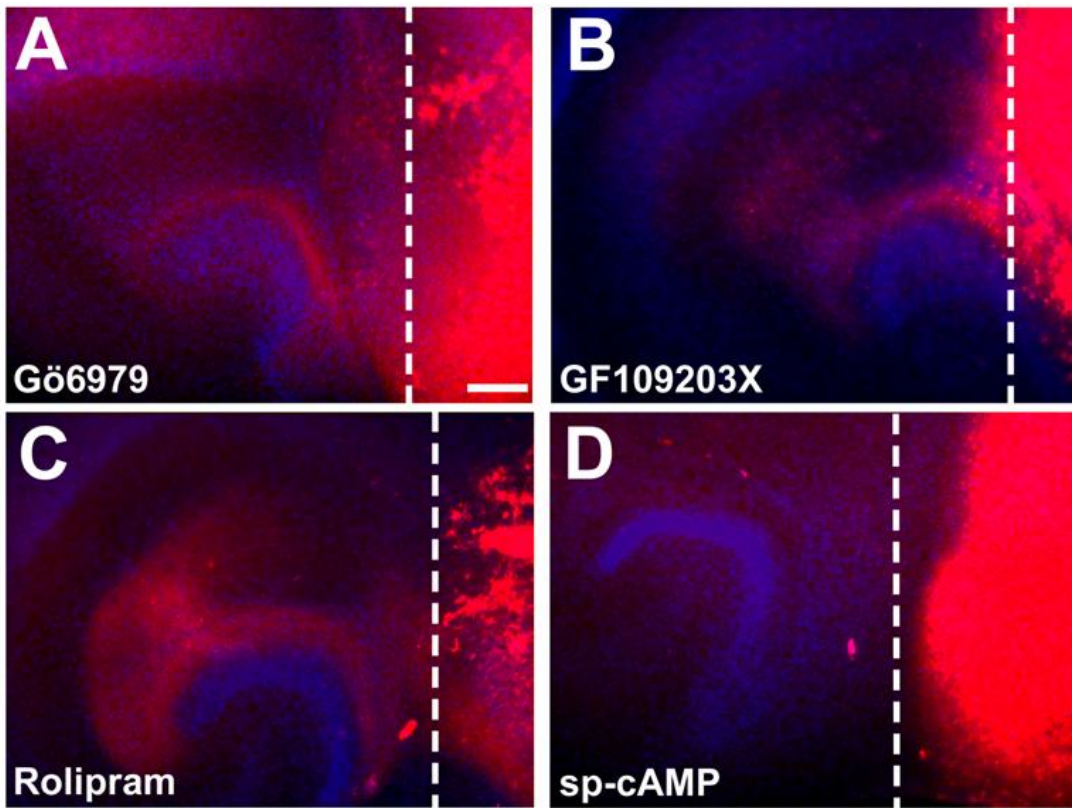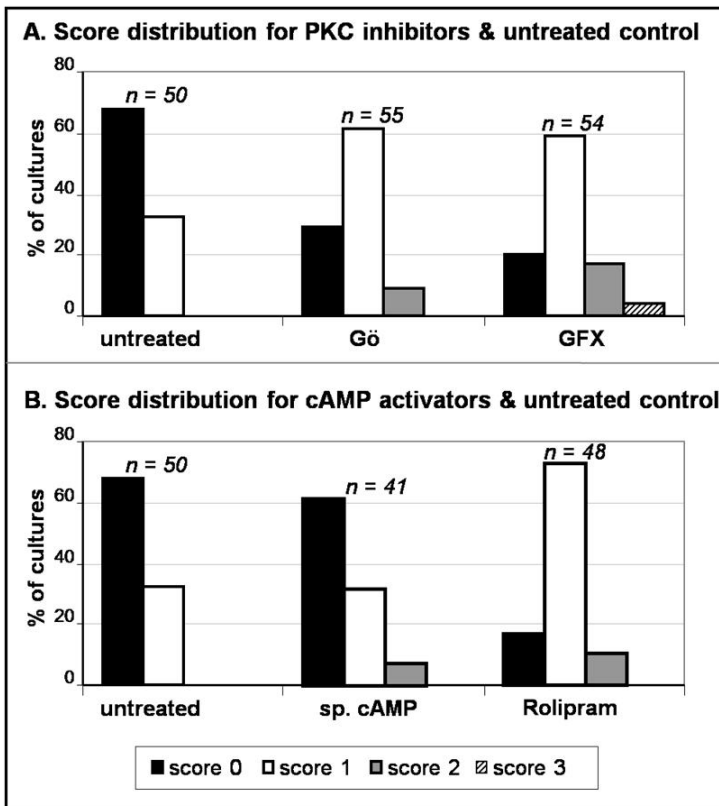
3.4.4   Treatment with cAMP Activators

We have used two drugs which are active in the cAMP signalling pathway: sp-cAMP is a membrane permeable active cAMP analogue, rolipram is an inhibitor of phosphodieaterase 4 (PDE4), the enzyme degrading cAMP. Both compounds are expected to increase cAMP activity. We found a significant increase (P<0.01; **) in regenerating fibers for cultures treated with Rolipram (*n*=48) while cultures treated with sp-cAMP (*n*= 41) did not show any significant increase (P < 0.05; *ns*) in the number of regenerating fibers (Fig. 3.3 B). In most of sp-cAMP-treated cultures there was no fiber growth across the lesion and in some of these cultures there was a limited growth with <50 fibers crossing the lesion site (Fig 3.4 B).

3.4.5   Other treatments

In addition we have tested 5 compounds working on various signalling systems. Pertussis toxin is an inhibitor of proteins which has previously been shown to promote axonal growth in the presence of inhibitory signals from the environment and was previously shown to promote regrowth of entorhinal fibres (Prang P, 2001). It also had a significant effect (p<0.001, n=42) in our experiments (Fig. 3.5 A, Fig. 3.6). Xestospongine C is an IP3-receptor blocker, inhibiting IP3-mediated Ca^{2+}-release from intracellular stores. Also with Xestospongine C we found an improvement of growth of entorhinal fibers across the lesion (Fig. 3.5 B, Fig. 3.6) which was significant with p<0,001 (*n*= 61). Two compounds working on the ras-MAPK pathway and on the PI3-kinase/AKT pathway also showed improved growth after the lesion paradigm. With LY27003, an inhibitor of PI3-kinase we found improved crossing of the lesion (Fig. 3.5 C, Fig. 3.6) with p<0.001 (n=53). A considerable number of XSp and LY treated cultures had >100 fibers crossing through the lesion. A certain promotion of growth was found with FTSCA, an inhibitor of ras, however the differences did not reach statistical significance (Fig. 3.6, p>0.05, n=57). The compound Y27632 which is an inhibitor of ROCK and supposed to inhibit the activity of Rho did not show any improvement of fiber growth (Fig. 3.5D, Fig. 3.6, p>0.05, *n*= 41).
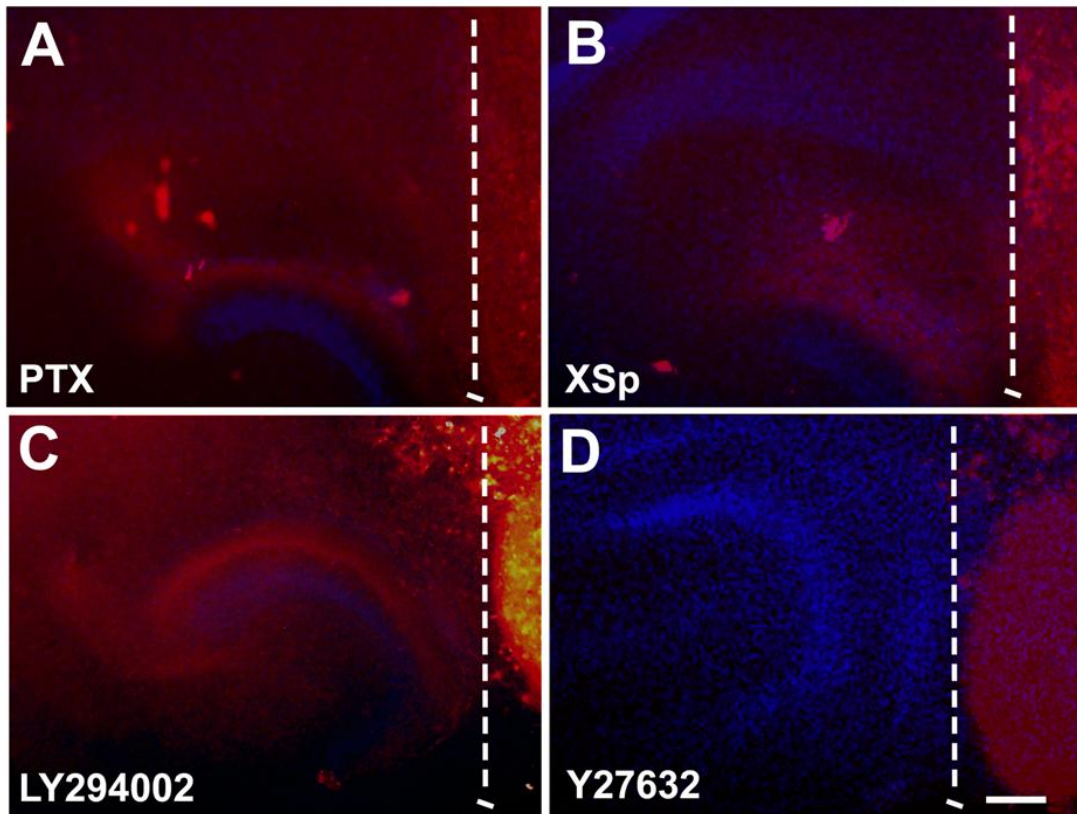
Fig3.5 Other treatments. Pertussis toxin (A), Xestospongin C (B) and LY294002 (C) show significant increase in regeneration after treatment; and Y27632 (D) which does not show any improved regeneration after treatment. Scale: 100µm
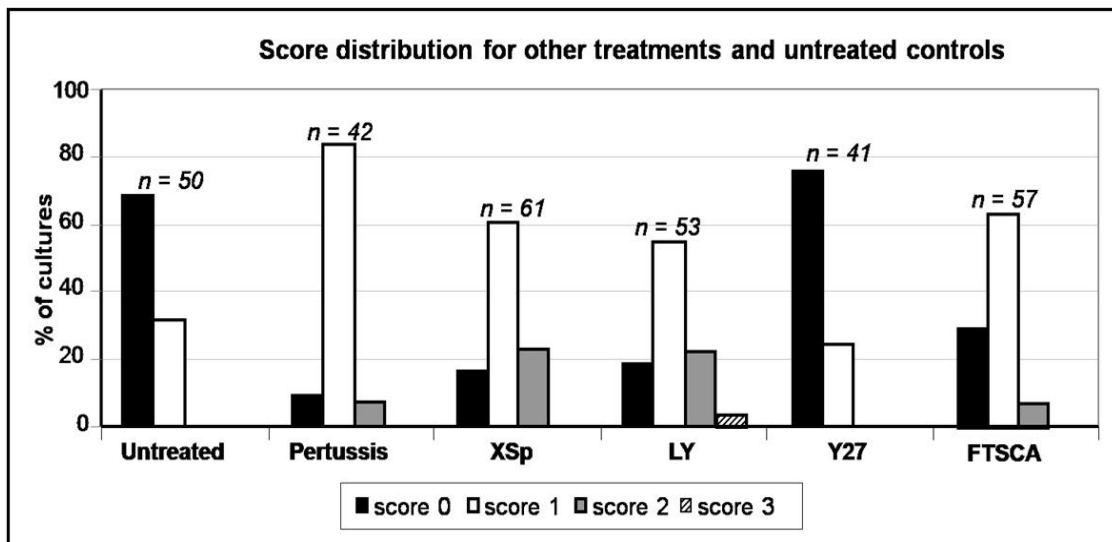
Fig. 3.6 Score distribution for treated cultures

3.5   Discussion

We have tested the potential of several compounds known to act on different signal transduction pathways to promote axonal growth and target re-innervation after mechanical lesions in entorhino-hippocampal slice cultures. We find that many of these compounds had a promoting effect on fibre re-growth, whereas others showed no effect. Our results demonstrate that pharmacological compounds affecting signal transduction pathways are promising drug candidates for promoting axonal repair in the central nervous system.

3.5.1   Regeneration of entorhinal fibres in organotypic slice cultures

Entorhino-hippocampal slice cultures have been used in the past for assaying regeneration of entorhinal fibres towards the dentate gyrus (for a recent review and protocol see Del Turco D, 2007). It was shown originally by Li et al. (Li D, 1993) that entorhinal fibre regenerate in slice cultures and terminate with correct target specificity in the dentate gyrus. This regeneration is age-dependent and is lost with maturation of the slice culture (Woodhams PL, 1993; Li D, 1995; Prang P, 2001). It was shown that the regeneration of the entorhinal fibres could be stimulated by growth factors (Woodhams PL, 1996). Even in mature slices regeneration of entorhinal fibres could be induced by treatment with growth factors and pharmacological compounds (Prang P, 2001). Furthermore, regeneration of entorhinal fibres was shown to be dependent of the presence of Cajal-Retzius cells (del Rio JA, 2002). Regeneration of entorhinal fibres in matured cultures could be achieved by a combined digestion of CSPG with Chondrotinase ABC and the neutralization of NOGO inhibitors (Mingorance A, 2006) whereas overexpression of Bcl-2 had no beneficial effect on regeneration (Solé M, 2004). In this study we have tested a number of pharmacological compounds affecting signalling pathways for their potential to induce re-growth of entorhinal fibres after mechanical lesions. Our findings that some of these compounds do promote re-growth, that such compounds which have been shown to be active previously also worked in our experiments whereas some other compounds had no effect confirm that this slice culture model is well suited for the testing of potential treatment strategies aimed at improving axonal regeneration.

3.5.2 Promotion of regeneration by PKC inhibitors and cAMP activators

The role of PKC activation for axonal growth is not fully clear. PKC is phosphorylating GAP-43 which a major growth associated protein (He Q, 1997). A certain PKC activation is such necessary for axonal outgrowth (Kolkova K, 2000) and treatment with PKC inhibitors was shown to induce growth cone collapse and stop axonal growth (Théodore L, 1995; Heacock AM, 1997). More recently it was shown that chemorepulsion of cerebellar granule cell axons by netrin-1A could be converted to attraction through activation of protein kinase C (Bartoe, 2006). Despite this growth promoting activity of PKC it was shown that chronic inhibition of PKC promotes axonal regeneration both in organotypic slice cultures (Prang P, 2001) and after spinal cord injury in vivo (Sivasankaran R, 2004). These findings have been confirmed in this study. Two different inhibitors, Gö6976 and GF29003X, used in this study yielded a very similar promotion of regeneration of entorhinal fibres. It is likely that the mechanism of action is through the blockade of inhibitory signals from the environment (myelin inhibitors, chondroitin sulphate proteoglycan) as suggested by Sivasankaran et al. (2004). Apparently the chronic exposure to the inhibitors is still compatible with the functioning of the growth machinery of the growing fibres but is sufficient to abrogate negative influences from the environment. This is also supported by a recent study showing that inhibition of PKC prevented growth cone collapse induced by exposure to an inhibitory signal in cultured PC12 cells (Conrad S, 2007).

The elevation of cAMP levels in the axon and growth cone is well known for stimulating axonal growth. Actively elongating growth cones turn towards a cAMP source (Song HJ, 1997) and many of the neurite outgrowth promoting effects of neurotrophins are thought to be mediated by cAMP as first suggested by (Roisen FJ, 1972). As for axonal regeneration, the major effect of an elevation of cAMP levels is thought to allow growth cones to overcome inhibitory signals from CNS myelin (for review see Hannila SS, 2008). The elevation of cAMP levels has been shown to promote axonal growth on inhibitory myelin substrates in vitro (Cai D, 1999). Furthermore, when compounds known to elevate cAMP levels were used after spinal cord injury in vivo they were able to promote axonal regeneration (Qiu J, 2002) through the induction of CREB (Gao Y, 2003). Treatment with the phosphodieasterase inhibitor rolipram was shown to improve axonal growth after spinal cord injury (Nikulina E, 2004) in rats. We have used this compound in this study and have also found a promoting effect on

regeneration. Surprisingly, sp-cAMP a membrane permeable cAMP analogue which should have similar actions compared to rolipram was not effective in our experiments. There are several possible explanations for this difference. Sp-cAMP may not have diffused efficiently in the depth of the slice culture and have reached efficient concentrations. Alternatively, the chronic elevation of cAMP levels required for the induction of axonal growth may not be achieved by treatment with analogues, but may rather require the use of an inhibitor of degradation such as rolipram.

3.5.3 Promotion of regeneration by inhibitors of other signalling pathways

Pertussis Toxin inhibits Gi- and Go-proteins thus interfering with signal transduction through G-protein coupled receptors (Post GR, 1996). It has been shown previously that pertussis toxin treatment prevents growth cone collapse in response to myelin-associated inhibitors (Igarashi M, 1993). When tested in the entorhino-hippocampal slice culture system, it has been shown previously to be strong inducer of axonal regrowth (Prang P, 2001). The results from this study confirm these findings. The good potential of pertussis toxin to promote axonal re-growth is an indication that inhibitory signals from the environment are an important determinant of regeneration in this system.

Xestospongin C is an IP3-receptor antagonist which prevents IP3-mediated Ca^{2+}-release from intracellular stores (Gafni J, 1997) and is thus part of the phospho-inositol pathway which also leads to activation of Protein kinase C. Surprisingly, this receptor has not been implicated in axonal growth so far, although it has been reported to be strongly expressed in neurons during axogenesis (Nakanishi et al 1991; Dent et al. 1996). Our finding that blocking the IP3-receptor promotes axonal re-growth in our model system is compatible with the growth promoting effect of inhibitors of protein kinase C, since both are part of a related signalling pathway. That inhibition of the phospho-inositol pathway at two different points is effective in fostering axonal re-growth in our lesion model clearly implies this pathway in the control of axonal regeneration after a mechanical lesion.

LY294002 is a blocker of PI3 kinase. The Pi3 kinase pathway has been frequently implicated in axonal growth and neuronal development (for review see Rodgers EE, 2002). Interestingly, in mice with a neuronal inactivation of PTEN, an inhibitor of PI3 kinase resulting in increased

activity of the PI3 kinase pathway a phenotype of neuronal hypertrophy involving enlargement of axonal tracts developed indicating that PI3 kinase activation will promote axonal growth (Kwon CH, 2006). Given these findings it is surprising that in our experiments a blocker of the PI3 kinase pathway was stimulating axonal re-growth. The reasons for this difference are not entirely clear. While it was shown that acute treatment of sensory growth cones with LY294002 had a collapsing effect on growth cones, these growth cones recovered rapidly and resumed outgrowth in the continued presence of LY294002 (Chadborn NH, 2006). Chronic LY294002 treatment as applied in our cultures might desensitize the PI3K/Akt pathway and make neurites unresponsive to signals normally inducing inhibition of axonal growth through inhibition of the PI3K/Akt pathway.

FTSCA is an antagonist of ras and suppresses ras-mediated activation of the MAP kinase pathway (Levitki 1996). Activation of ras and the MAP kinase pathway were reported to be required for neurite outgrowth (Kolkova K, 2000; Sarner S, 2000). Interestingly, downregulation of ras appears to be required for the induction of Eph mediated neurite retraction in neuroblastoma cells (Elowe S, 2001). In our experiments chronic FTSCA treatment had a promoting effect on fiber regeneration which did not reach statistical significance. Possible mechanisms of action might be similar as suggested for inhibition by LY294002, i.e. the chronic treatment might desensitize the neurites for inhibitory stimuli, resulting in a net increase of axonal growth.

Y-27632 is an inhibitor of ROCK, the downstream effector of RhoA and has been reported to promote functional recovery and axonal regeneration after spinal cord injury (Dergham P, 2002; Fournier AE, 2003). In another more recent study, Y-27632 was found to promote sprouting but not regeneration of fibres in the spinal cord (Chan CC, 2005). Inhibition of RhoA activity by application of the C3 peptide was also shown to be unable to induce axonal regeneration in vivo (Fournier AE, 2003; Sung JK, 2003). Furthermore, in culture experiments, Y-27632 treatment was shown to induce the expression of inhibitory chondroitin sulfate proteoglycans in astrocytes. We found no promotion of fibre re-growth in our slice culture model confirming that Y-27632 may not be a universal stimulator of axonal regrowth after CNS injury

3.6   Conclusions

Using a slice culture model that allows assessing axonal regeneration after a mechanical lesion we found that several pharmacological compounds did promote the re-growth of fibres through the lesion site. In the light of studies from other laboratories the inhibition of G-protein mediated signal transduction and protein kinase C activation are the most promising strategies for identifying novel drug candidates for the promotion of axonal regeneration. Our finding that inhibition of the IP3-receptor and of PI3-kinase promoted re-growth of entorhinal fibres might offer new therapeutic opportunities. Our findings confirm that targeting signals transduction pathways is a promising strategy to promote axonal growth after CNS lesions.

4. SPINAL CORD:

Spontaneous regeneration of intrinsic spinal cord axons in a novel spinal cord slice culture model

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4.1   Abstract

A substantial problem in research concerned with axonal repair is the use of a wide variety of lesion models and the complexity associated with the respective *in vivo* lesion paradigms. Organotypic slice cultures are a potential in vitro alternative because the cytoarchitectonic tissue organization is well preserved and the slices can be maintained in culture for several weeks. Until now no spinal cord slice culture model for the study of axonal growth has been available. Here we present a spinal cord slice culture model which is well suited for the study of axonal growth. The spinal cord slices were cut not in the transverse but in the sagittal longitudinal plane such that several spinal cord segments were included in the slice culture. In these cultures the typical ventro-dorsal polarity of the spinal cord was maintained and intrinsic spinal cord axons formed a strong fiber tract extending along the longitudinal axis of the slice. The axons became myelinated during the culture period and synaptic contacts were present in these cultures. After mechanical lesions the intrinsic spinal cord axons had a substantial potential for axonal growth and regeneration. The number of regenerating axons crossing the lesion site decreased with increasing maturation of the culture but even in mature cultures a small number of crossing fibers were present. This slice culture model could provide an important tool for several aspects of spinal cord research in the fields of axonal growth and regeneration, synapse formation, formation of intrinsic spinal cord circuits and myelination.

4.2   Introduction

Axonal regeneration after lesions does not occur in the mature central nervous system (CNS). In recent years several molecules involved in the control of axonal growth in the CNS were identified, and a number of possible strategies to enhance axonal regeneration and eventually treat spinal cord injury have been proposed (for reviews see Maier IC, 2006; Liu BP, 2006; Zhou FQ, 2006; Busch SA, 2007; Crespo D, 2007). A substantial problem in research concerned with axonal repair is the use of a wide variety of lesion models and the complexity associated with the respective *in vivo* lesion paradigms (Steward O, 2003). Unfortunately, cell and tissue culture studies are only of limited value for the study of axonal regeneration in the CNS. The important role of molecules and factors residing in the microenvironment of the lesioned

fibers can only be studied in intact tissue, and not in dissociated cells or explants. This is different for organotypic slice cultures (Gähwiler, 1981; Stoppini L, 1991; Kapfhammer, 2005) which have been previously used for the study of regeneration *in vitro*. Within these cultures the cytoarchitectonic tissue organization is well preserved, and the slices can be maintained in culture for several weeks. In entorhino-hippocampal cultures, axonal regeneration was found to be abundant when the lesion is made up to 4 days in vitro (DIV), but is greatly reduced after lesions made at DIV 6 or later (Prang P, 2001). In a similar approach, the repair of the entorhino-hippocampal projection by transplantation of immature neural tissue has been studied (Radojevic V, 2004). While these models are of value for identifying factors which might improve axonal regrowth within the CNS, most research on axonal regeneration is focused on the spinal cord, as spinal cord injury is the most common and serious cause of an axonal lesion in human patients. We have now studied the possibility to use organotypic slice cultures of spinal cord cut in the longitudinal sagittal plane to assess axonal growth and regeneration in vitro within a spinal cord microenvironment. Our findings show that these cultures maintain many aspects of the intact spinal cord. Furthermore we demonstrate that intrinsic spinal cord axons present in these cultures are capable of spontaneous regeneration in an age-dependent manner. Our findings suggest that spinal cord neurons retain a remarkable plasticity and that sagittal spinal cord cultures are an attractive *in vitro* model system for the study of spinal cord development and maturation and for axonal regeneration in the spinal cord.

4.3   Materials and Methods

4.3.1   Organotypic Slice Cultures

Animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were reviewed and approved by Swiss authorities. Spinal cord was dissected from postnatal (P0–P6) CB6F1 mice. Mouse pups were decapitated; the spinal cord was rapidly dissected out and was placed in cold preparation medium (MEM with 2mm glutamax; PH 7.3).Longitudinal sagittal slices of cervical spinal cord were cut using a tissue chopper (McIlwain) at a thickness of 350µm and transferred onto a

permeable membrane (Millicell-CM, Millipore, Zug, Switzerland; Fig. 4.1). Cultures were incubated in incubation medium (50% MEM, 25% basal medium eagle, 25% heat inactivated normal horse serum, 0.65% glucose and 2mM glutamax; pH 7.3) at 37ºC in humified air with 5% CO_2. The incubation medium was changed every second day. Cultures were incubated for up to 4 weeks.
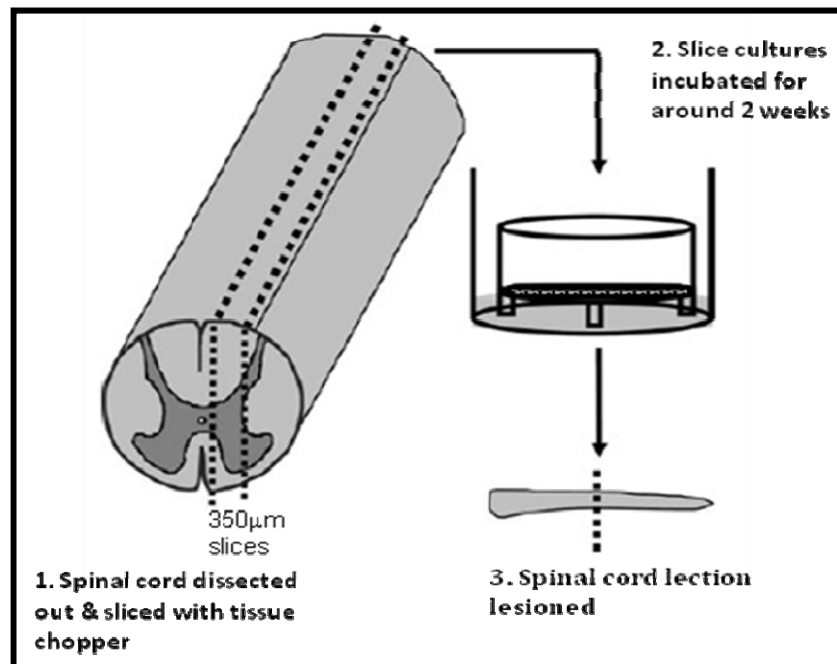
Fig.4.1 Schematic representation of method used for making organotypic slice cultures of longitudinal sagittal slices of spinal cord. Longitudinal sagittal slices (350µm thick) were cut from a piece of cervical spinal cord, transferred to a culture insert and cultured for up to 4 weeks. A mechanical lesion was performed by a complete cut through the slice culture with a scalpel blade.

4.3.2 Treatment with Rolipram

To test the effectiveness of the phosphodiesterase 4 inhibitor rolipram for improving growth across the lesion site a lesion was made after 6 days in vitro in cultures derived from neonatal (P0) mouse pups. Rolipram (Tocris, Bristol, UK) was used at a concentration of 0.5µM. Treatment started on the day of lesion and continued until the cultures were fixed 6 days after lesion. Treated cultures were compared to untreated controls from the same experiment.

4.3.3 Immunohistochemistry and microscopy

Cultures were fixed with 4% paraformaldehyde (PFA) for 2 h at room temperature (RT). For immunohistochemical staining cultures were washed 3 times for 10 minutes with phosphate buffered saline (PBS), and blocked with a solution containing 0.5% Triton X-100 and 3% normal goat serum (NGS) for 20 minutes prior to the primary antibody. The following primary antibodies were used: mouse anti-neurofilament SMI31 (Covance, Princeton NJ, USA; 1: 1000), mouse anti-neurofilament SMI32 (Covance, Heidelberg, Germany; 1:1000), mouse anti-MBP SMI94 (Covance; 1:500), rabbit anti-Calretinin (Swant, Bellizona, Switzerland; 1:1000), rabbit anti-synaptohysin (Synaptic Systems; 1:500), monoclonal mouse anti-MPZ (clone P07; Archelos, 1993), and monoclonal mouse anti-MOG (clone Z12; Piddlesden SJ, 1993). Antibodies were diluted in PBS and applied either for 2 h at room temperature or overnight at 4°C. Sections were then washed 3 times for 10 min with PBS and incubated with the secondary antibodies goat anti-mouse Alexa 488 (Molecular Probes; 1: 500), goat anti-rabbit Alexa 546 (Molecular Probes; 1: 500) for 2 h at RT. Sections were again washed twice with PBS, and mounted with Mowiol. Cultures were analyzed using an Olympus AX-70 fluorescence microscope and pictures were taken using a Spot digital camera. The brightness and contrast of the pictures was adjusted using Photoshop image processing software.

For confocal microscopy, immunostained slices were analyzed with a confocal laser scanning microscope (TCS4D; Leica Microsystems, Mannheim, Germany) equipped with an Ar-Kr-laser and an acousto-optical device (acousto-optic tunable filter module). Images were recorded with a 100x lens. After processing with a median filter to suppress background, image stacks were analyzed and reconstructed using the Imaris software package (Bitplane, Zurich, Switzerland).

4.3.4 Semiquantitative evaluation of regeneration

For evaluation of regeneration cultures were viewed with the 20x objective. Because the number of fibers was too large to be counted individually, a semiquantitative evaluation of fiber growth through the lesion was done. The fiber density on both sides of the lesion was inspected and an estimation of the percentage of the fibers running through the centre of the

lesion site was made. In cultures without a lesion this would be 100%. The amount of fibers crossing through the lesion site was estimated and a score was assigned to each slice according to the scoring system shown in Table 4.1. Individual scores were determined for at least 20 slices from at least 3 independent experiments for each data point which shows the mean of the individual scores and the standard deviation. The statistical significance of differences in parameters was assessed by Mann-Whitney's nonparametric test using Graph Pad Prism software (San Diego, CA, USA).

Score	Meaning
6	>75% of fiber crossing through lesion
5	50 – 75% of fiber crossing through lesion
4	25 - 50% of fiber crossing through lesion
3	10 - 25% of fiber crossing through lesion
2	<10% of fiber crossing through lesion
1	Occasional fibers crossing through lesion
0	No fibers crossing through lesion

Table 4.1   Score system for fiber counts crossing through the lesion.

Score system for counts of fibers crossing the lesion. The lesion site was viewed at high magnification in cultures immunostained with SMI31 and the number of fibers crossing the lesion site was determined and related semiquantitatively to the total number of fibbers present proximal to the lesion site.

4.3.5   Cryosections

CB6F1 mice (P8 and P14) were sacrificed with an overdose of pentobarbital and perfused through the left ventricle with PBS with Heparin (50 IU/ml), followed by 4% PFA as fixative. The spinal cord was dissected and fixed in PFA overnight at 4°C. The spinal cord was then transferred to 30% sucrose overnight for cryoprotection and frozen in isopentane at −40°C. After embedding in tissue freezing medium (Jung, Germany), 25μm cryosections were cut. Immunostaining and microscopy of the sections was carried out as described above for the slice cultures.

4.4   Results

4.4.1   Sagittal spinal cord slice cultures maintain ventro-dorsal polarity

Spinal cord is typically viewed in the transverse plane for which the cytoarchitectonic organization is well described with the layers of Rexed (Rexed, 1954). Because in the early postnatal spinal cord these layers are present throughout most of the medial to lateral extension of the spinal cord grey matter, in the sagittal slices the dorsal layers will be on one half and the ventral layers on the other half, i.e. the slices originally will have a similar ventral-dorsal polarity as a transverse slice, with dorsal located at the top and ventral located at the bottom. We have tested whether this polarity is maintained over prolonged culture periods by immunostaining for dorsal and ventral markers.

Calretinin stains neurons throughout the spinal cord, but shows a dense labelling of small neurons in layer II (Rexed, 1954). Labelling of non-phosphorylated neurofilaments with SMI32 has been shown to be a reliable marker for motoneurons (Carriedo SG, 1996; Tsang YM, 2000; Rao SD, 2003; Avossa D, 2006). In addition, SMI32-positive cells in our cultures were also positive for vesicular acetylcholine transporter (VAChT), another marker for motoneurons (Fig. 4.2).
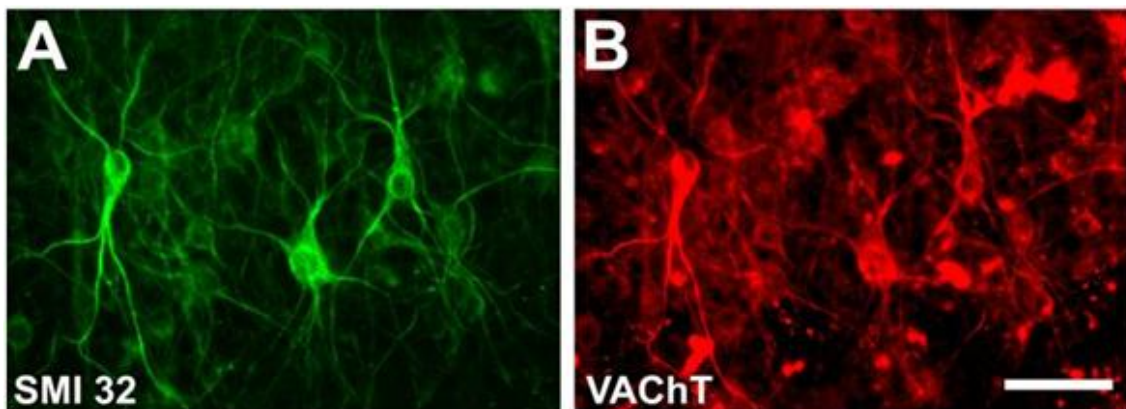

Fig. 4.2   Double staining of motoneuron like cells in the cultures with SMI 32 and VAChT.

A. SMI32 positive cells present in the ventral half of the culture showing motoneuron-like morphology. B. The same cells are also positive for VAChT, a motoneuron marker, confirming the identity of the SMI32-positive cells as motoneurons. Scale bar = 50μm

We have confirmed these staining patterns in sagittal cryosections of P14 mouse spinal cord, in which the double staining clearly indicates the ventrodorsal polarity (Fig. 4.3 A). A similar staining pattern was observed in P6 sagittal spinal cord slices cultured for 12 days. The calretinin-positive dorsal domain is somewhat increased in thickness, but is otherwise well preserved (Fig. 4.3 B). The same applies to the ventral domain with the motoneurons, which can be clearly identified in the cultures by SMI32 staining. In higher magnification, motoneurons with an extensive dendritic tree and axonal processes can be identified, indicating good survival and differentiation of these cells (Fig. 4.3 C). Taken together these findings indicate that in sagittal slice cultures there is good neuronal survival and differentiation and the ventro-dorsal polarity of the spinal cord is preserved in these organotypic slice cultures after 2 weeks in vitro.
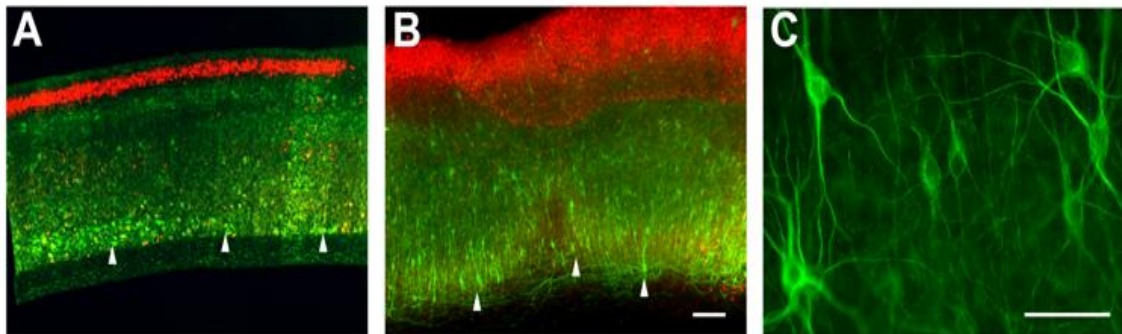
Fig.4.3   Comparison of the spinal cord slice culture with the spinal cord in vivo.

A. Cryosection of a P14 mouse spinal cord, stained for SMI32 in green and Calretinin in red. Many SMI32 positive motoneurons are present in the ventral part of the culture (arrowheads). The Calretinin-positive small neurons are concentrated in layer II.   B. Longitudinal slice culture after 12 days in vitro. The calretinin-positive neurons are distributed in the dorsal part of the culture. SMI32-positive motoneurons are present in the ventral part of the culture (arrowheads). The overall organization of the spinal cord is similar to that of the section. Scale bar = 200µm C. High magnification of the morphology of SMI32-positive motoneurons from a spinal cord culture. Scale bar = 50µm.

4.4.2   Neurofilament staining reveals longitudinally extending fibers in spinal cord cultures.

Motoneurons positive for SMI32 staining extended long axons which ran in the longitudinal direction of the spinal cord in the area where normally the ventral columns would be located (Fig. 4.4 A). Since spinal motoneurons do not normally project within the spinal cord we assume that these fibers are motoneuron axons running aberrantly in the ventral part of the cultured spinal cord.
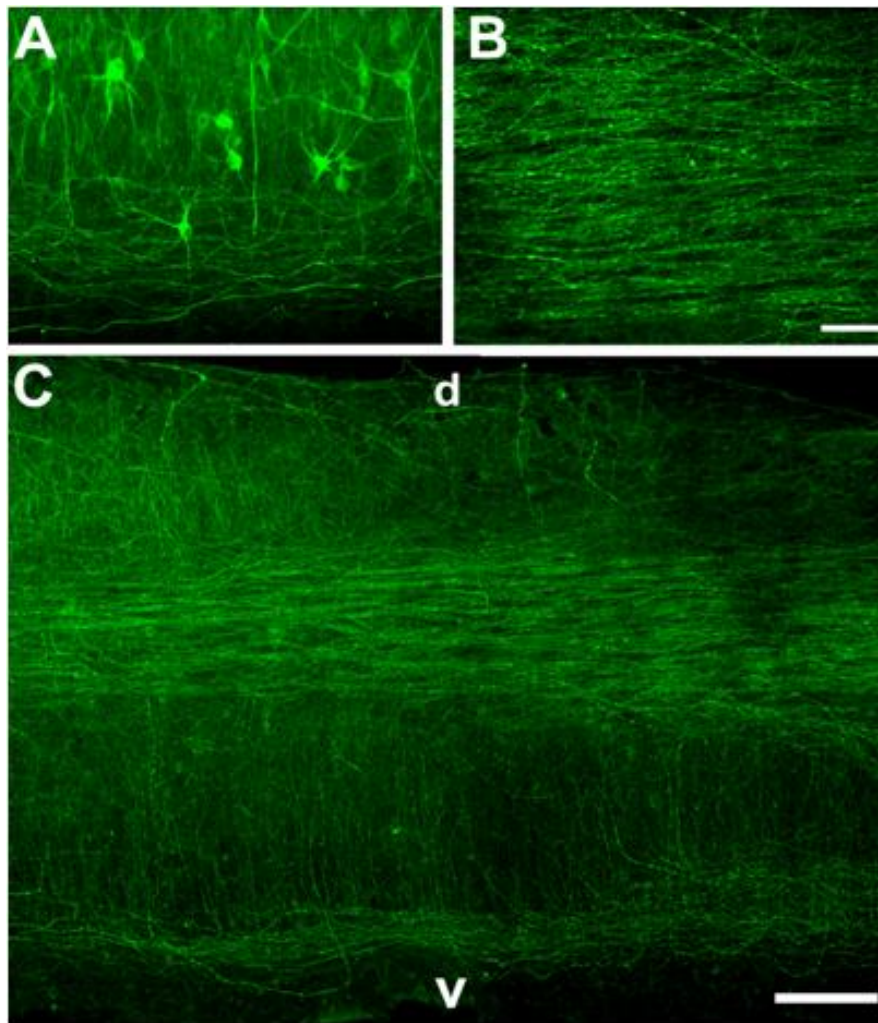
Fig. 4.4 Axonal processes develop in longitudinal spinal cord slice cultures.

A   Motoneurons present in the ventral part of the culture identified with SMI32 give rise to a fiber bundle of motoneuron axons in the ventral part of the culture. B. High power view of a SMI31-positive bundle of intrinsic spinal cord fibers in the central part of the culture at DIV 10. Scale bar = 50µm for A and B. C. Composite picture of a spinal cord culture stained with SMI31 at DIV 10. Note the strong fiber bundle in the central part of the culture (compare with B), and the loose fibers in the ventral domain which are likely to represent motoneuron axons (compare with A)

In addition to the axonal processes from motoneurons, staining with the antibody SMI31 which labels mostly axonally located phosphorylated neurofilaments revealed the presence of an extensive fiber bundle running in the longitudinal direction in the central part of the spinal cord culture (Fig. 4.4 B, C). After few days in vitro they typically formed a strong fiber bundle as seen in Fig. 4.4 B, C. This fiber bundle was present from the rostral to the caudal end of the explants. Because SMI31 stains only the processes, the cells of origin of these fibers could not

be clearly identified. However, since it is known that propriospinal neurons form ascending and descending connections extending over several spinal cord segments (Nandi KN, 1993), (Dutton RC, 2006) it is likely that this bundle contains both ascending and descending axons originating from propriospinal neurons.

4.4.3 Synaptic contacts and myelination in spinal cord cultures

In order to evaluate the presence of synaptic contacts in the cultures we have done immunostainings with the presynaptic marker synaptophysin. These showed intense staining throughout the culture (Fig. 4.5 A). In higher magnification, punctate staining along dendrites of SMI32-labeled motoneurons became evident (Fig. 4.5 B). This was confirmed by confocal microscopy which confirmed the presence of synaptophysin-positive boutons along the dendrites of SMI32 positive cells (Fig. 4.5 C).
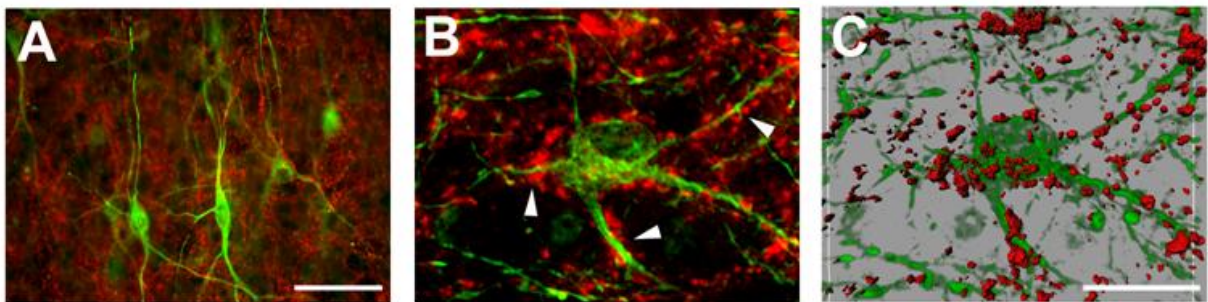
Fig. 4.5 Synaptophysin staining suggests the presence of synapses around the cell body of SMI32-positive motoneurons. A Synaptophysin immunoreactivity was present throughout the culture and could be sometimes localized around the dendrites of SMI32-positive motoneurons. Scale bar = 50μm. B Confocal plane of a SMI32-positive cell with synaptophysin-positive punctae (arrowheads) around the dendrites. C 3D-reconstruction of the cell shown in B confirming the presence of synaptophysin-positive punctae around the dendrites. Scale bar = 20μm.

Myelination of fibers within the spinal cord cultures was analyzed using the antibody SMI94 directed against MBP. When cultures were stained after short cultivation periods of 2 to 4 days very few myelinated fibers were present in the cultures, but several SMI94 positive cells were visible. When these cells were viewed in high magnification, they had the typical morphology of myelinating oligodendrocytes (Fig. 4.6 A, B). One cell typically was myelinating several axons (arrowheads in Fig. 4.6 A, B). The identity of the myelinating cells was confirmed with an

oligodendrocytes-specific antibody against myelin oligodendrocytes glycoprotein (MOG) which stained the myelinating cells (Fig. 4.6 A) and the myelinated axons. In contrast, no staining of myelinated fibers was observed with an antibody directed against P0, the major protein in PNS myelin (data not shown) indicating that myelin formation in the cultures was by oligodendrocytes and not by immigrated Schwann cells. After one week in vitro, oligodendrocytes could still be identified by SMI94-staining, but now many myelinated fibers were present in the culture (Fig. 4.6 C). After 2 weeks in vitro, many fibers of the central fiber bundle (see above) were now SMI94 positive, indicating an extensive myelination of these fibers (4.6 D). It is noteworthy that the development and the myelination of the intrinsic spinal cord fibers is taking place completely within the slice cultures, as this fiber tract was not evident at the time of explantation.
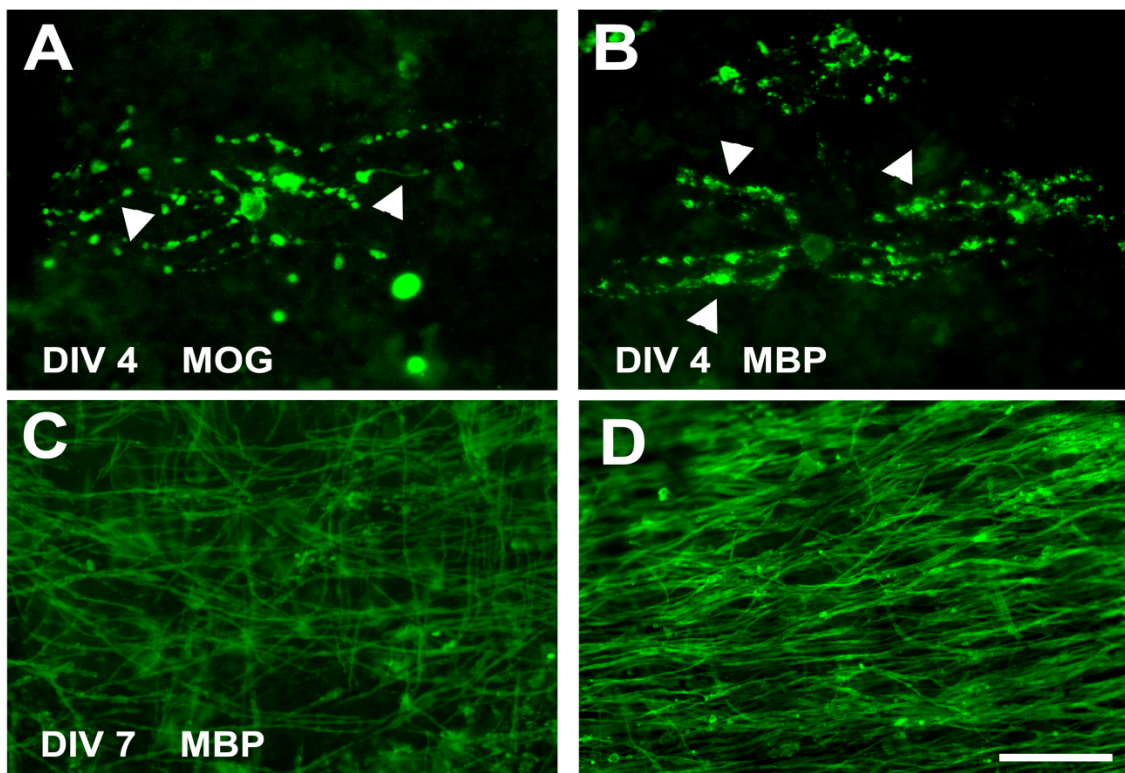
Fig. 4.6 Formation of CNS myelin in longitudinal P0 spinal cord slice cultures with increasing time in vitro.

A. MOG-positive oligodendrocyte in a spinal cord culture at DIV4 confirming the identity of the myelinating cells as CNS oligodendrocytes. B. MBP-staining of an oligodendrocyte present in the culture at DIV4. Note the typical morphology with one oligodendrocyte starting to ensheath several axons (arrowheads). C. At DIV 7 individual MBP-positive cells were difficult to identify, because the staining became concentrated in the processes. Many MBP-positive fibers were now visible throughout the culture. D. At DIV14 dense bundles of myelinated fibers were present in the culture. Scale bar = 50μm.

4.4.4 After mechanical lesions, spinal cord fibers regenerate spontaneously

We have performed lesions of the spinal cord fibers by doing a transverse cut through the spinal cord culture with a scalpel blade. Lesions could be identified in the cultures by the absence of calretinin-positive cells throughout the lesion zone. We have done such lesions in cultures derived from P0 spinal cord after 6 days in vitro, and have analyzed the fiber tract one week after the lesion. In these cultures the lesion has a strong impact and the majority of the fibers stop at the lesion (Fig. 4.7A, B) which can be easily recognized as a gap in the continuous fiber bundle. Nevertheless at higher magnification many fibers crossing the lesion site can be identified (Fig. 4.7B). Under these conditions there obviously is a mixed response to the lesion with some fibers crossing the lesion site, but many fibers stopping at the lesion.
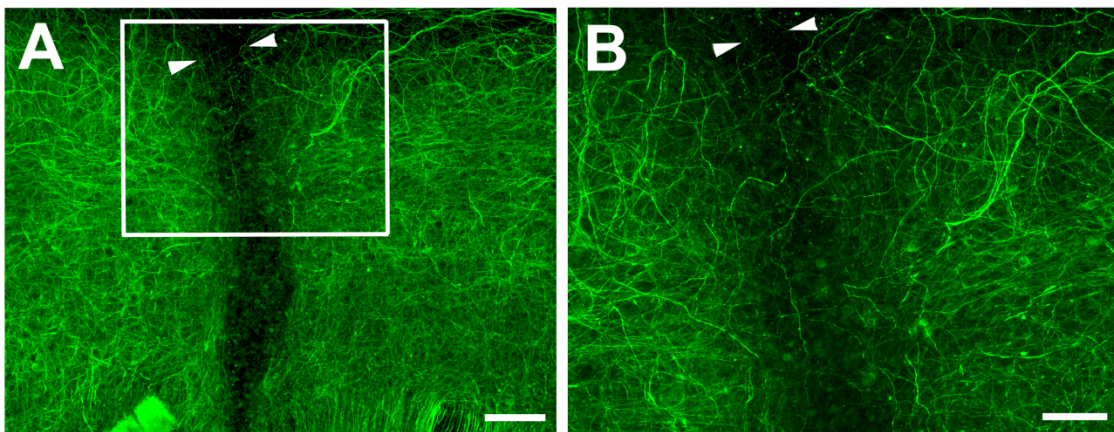
Fig. 4.7   After mechanical lesions, intrinsic spinal cord fibers cross the lesion site.

A. Low power view of the lesion site of a spinal cord culture from a P0 mouse with a lesion made at DIV6. Many fibers stop at the lesion site. Scale bar = 100µm.   B. At higher magnification, many fibers can be seen crossing the lesion. Lesions indicated with arrowheads, scale bar = 50µm

In order to see how the ability of the fibers to cross the lesion site is related to the maturity of the culture we have systematically varied both the age of the spinal cord at the time of establishing the culture and the culture period before doing the lesion. Lesions were performed on cultures derived from P0 mice after 0, 2, 4, 6, 8, and 10, and in some cases 12, 14 and 16 days in vitro (DIV). Cultures were fixed 5 days after lesion. Fiber growth was analysed by viewing with 20x and evaluated using a scoring system based on the number of fibers crossing the lesion (Table 4.1). Examples of the lesion sites from lesions made after varying time in culture are shown in Fig. 4.8. For lesions made after 2 days in vitro, a large

number of fibers could be seen crossing the lesion (Fig. 4.8 A, D), less fibers were crossing after a lesion made at 6 days in vitro (Fig. 4.8 B, E), and only few fibers were crossing after a lesion made at 12 days in vitro (Fig. 4.8 C, F). In the semiquantitative evaluation it becomes evident that there is a continuous decline in the number of fibers crossing the lesion with increasing maturation in culture (Fig. 4.9). It should be noted, however, that even with lesions made as late as 12 or 14 days in vitro there were some fibers crossing the lesion, i.e. there was no complete suppression of fiber growth through the lesion site.
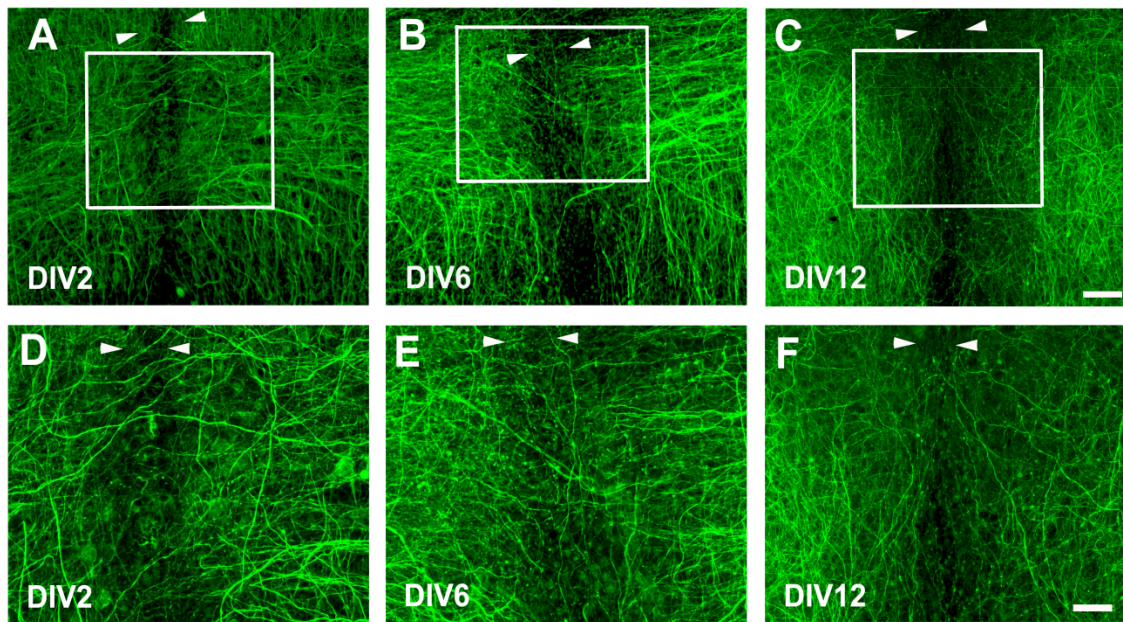
Fig. 4.8 With increasing time in culture, fewer fibers grow across the lesion site. Low power (upper row) and high power (lower row) view of the lesion site (arrowheads) of cultures with lesions after 2 (A, D), 6 (B, E) and 12 (C,F) days in vitro (DIV). **A. and D.** After lesions made at DIV2, many fibers cross the lesion site; **B. and E.** After lesions made at DIV6, less fibers cross the session site; **C. and F.** After lesions made at DIV12 only few fibers cross the lesion site. Lesions are indicated with arrowheads, scale bar = 100µm in (C for upper row) and = 200µm in (E for lower row).
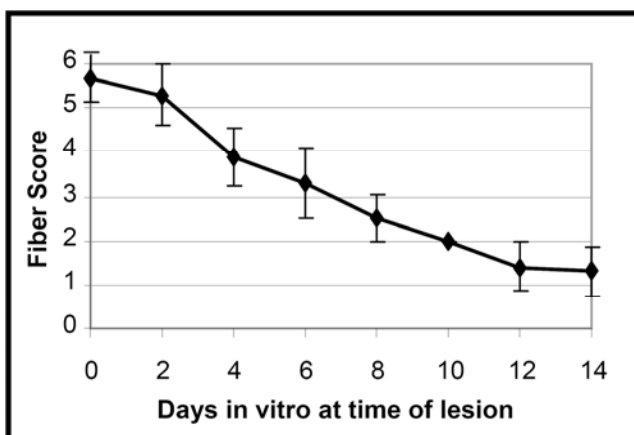
Fig. 4.9 Graph showing the crossing scores (y-axis) for lesions made after increasing time in vitro (x-axis) for P0 mice. Error bars show SD. The number of slices analyzed for the different time points were the following: n = 21, 24, 21, 19, 10, 14, 15, 13 for DIV 0, 2, 4, 6, 8, 10, 12, 14. With increasing time in culture, the number of fibers crossing through lesion decreases.

4.4.5 Comparable fiber growth in cultures derived from mice at postnatal ages P0 – P6

In order to evaluate the effect of the age of the mouse pups at time of culture we varied this age from P0 to P6 and always made the lesion after 6 days in vitro. In mice older than P6 no good spinal cord cultures could be established. Representative images of lesioned cultures are shown in Fig. 4.10. There was substantial crossing of fibers in cultures derived from all postnatal ages (Fig. 4.10 A – C). This is also evident from the semiquantitative evaluation, where there was a slight decrease in the scores with increasing age (Fig. 4.11). This difference was statistically not significant. This indicates that at least until explantation up to P6 the fibers in the spinal cord slices retain their potential to grow across the lesion site after a mechanical lesion.
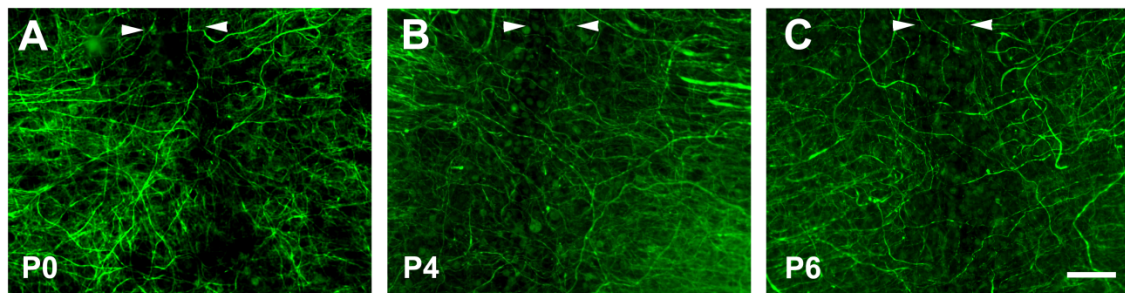
Fig.4.10 Until P6, fiber growth across the lesion site is only mildly affected by the postnatal age at the time of culture.

View of the lesion site after a lesion made at DIV4 in cultures derived from P0 (A), P4 (B) and P6 (C) mice. In cultures derived from all ages there is substantial crossing of SMI31 positive fibers through the lesion site. Lesion indicated with arrowheads, scale bar = 50µm.
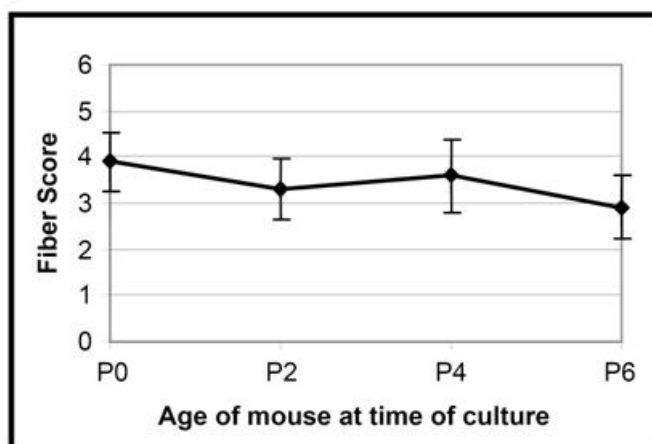
Fig. 4.11 Graph showing the crossing scores (y-axis) for lesions made at DIV4 in cultures derived from mice with increasing age (x-axis). The number of slices analyzed for the different time points were the following: n = 21, 18, 17, 22 for P0, 2, 4, 6. Error bars show SD. With increasing age, there is only a slight decrease in fiber crossing through the lesion.

4.4.6 Promotion of axonal growth through the lesion site can be induced by pharmacological treatment

As an in vitro system this culture model is an attractive tool for searching for pharmacological compounds which might be able to improve regenerative axonal growth through a spinal cord lesion. Elevation of cyclic AMP by the phosphodiesterase inhibitor rolipram has been previously shown to promote axonal regeneration in the spinal cord (Nikulina E, 2004). We have tested whether application of rolipram was able to stimulate axonal growth through the lesion site in the spinal cord cultures. We have found that application of rolipram induced a substantial and significant promotion of axonal growth through the lesion site (Fig. 4.12). This demonstrates that this culture system can be an attractive tool for evaluating known compounds and identifying novel substances for their potential to promote axonal regeneration in the spinal cord.
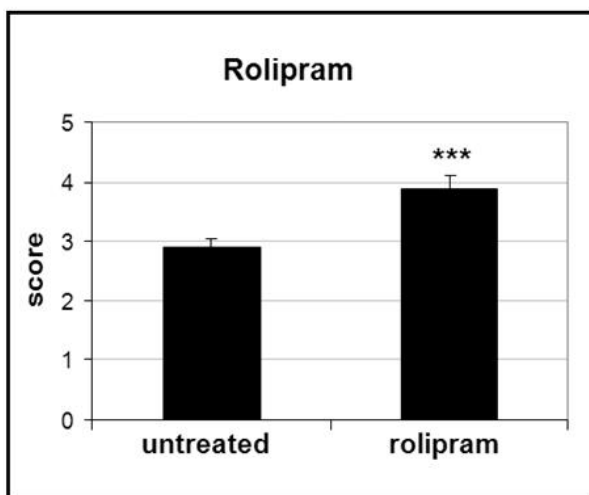
Fig. 4.12 Histogram showing scores of regenerating fibers through the lesion site for cultures treated with rolipram (n= 20) as compared to untreated controls (n= 19). Results were analysed by Mann-Whitney non-parametric test with 95% confidence limit. There is a significant increase (P<0.001; ***) in the fiber scores of rolipram treated cultures as compared to untreated controls.

4.5 Discussion

In this study we present a spinal cord slice culture model which is well suited for the study of axonal connections and axonal growth. The spinal cord slices were not cut in the transverse but in the sagittal longitudinal direction such that a slice includes several spinal cord segments. We have shown that in these cultures the typical ventro-dorsal polarity is maintained and that many axons are present extending along the longitudinal axis of the slice. Furthermore, we present evidence that the longitudinal axons are getting myelinated during the culture period and that synaptic contacts are present in these cultures. In an initial study we also show that

this culture model is suited for the study of axonal regeneration in vitro in a spinal cord microenvironment, and that intrinsic spinal cord axons have a substantial potential for axonal growth and regeneration after mechanical lesions. This slice culture model can provide an important tool for several aspects of spinal cord research in the fields of axonal growth and regeneration, synapse formation, formation of intrinsic spinal cord circuits and myelination.

4.5.1   Spinal cord slice cultures

Organotypic slice cultures of spinal cord have been used in neuroscience research for a long time (e.g. Crain SM, 1963; Braschler UF, 1989; Spenger C, 1991; Streit J, 1991). Classically, these cultures are derived from slices cut in the transverse plane. This way, many of the intersegmental connections of the spinal cord can either be preserved or can reform after slice explantation. In these cultures most of the major spinal cord cell types are present and spontaneous electrical activity develops similar to the situation in vivo (Rosato-Siri MD, 2004). In order to reflect the spinal cord microenvironment, the longitudinal cultures are required to maintain the basic cytoarchitectonic organization of the intact spinal cord. Because these slices are cut in the sagittal plane, most spinal cord cellular layers will be represented in the slice. However, in the sagittal slices it is not possible to identify the cytoarchitectonic layers unambiguously by a mere cellular staining because the layers are difficult to identify in sagittal sections. We have studied this aspect by immunostaining with SMI32, which in the spinal cord strongly labels motoneurons (Tsang YM, 2000) as a ventral marker, and with calretinin which stains intensely the small neurons of lamina II of the spinal cord (Ren K, 1993) as a dorsal marker. Our findings clearly show that the ventrodorsal polarity of the spinal cord is well maintained in the longitudinal slice preparation after a culture period of 2 weeks. This indicates that the cellular differentiation and cytoarchitectonic organization of the spinal cord neurons is similar to that of slice cultures of the transverse plane and reflects the in vivo situation well.

In addition to the transverse organization of neurons and connections in the spinal cord, there is also a marked longitudinal organization with many propriospinal connections between different segments of the spinal cord (Oppenheim RW, 1988; Dutton RC, 2006). One important aspect of the longitudinal slice culture is to maintain the cellular framework for the

preservation and restitution of such longitudinal projections. In our cultures, after few days *in vitro* a strong fiber bundle developed which extended in the longitudinal direction throughout the entire culture. Since no supraspinal tissue was present these fibers must represent intrinsic spinal cord connections, and might represent predominantly ascending and descending propriospinal fibers. In our culture model, unlike in the conventional spinal cord organotypic slice cultures, it would be possible to study intersegmental connections and other aspects of the longitudinal organization of the spinal cord. As the cultures represent the plane in which ascending and descending fibers in the spinal cord extend, it is particularly suited for the study of axonal growth and regeneration in the spinal cord.

We did not use cultures form animals older than P6 because in our hands neuronal survival in such cultures was more variable and a longitudinal fiber tract did not develop consistently. This is in agreement with transverse spinal cord slice cultures in which neuronal survival also appears to be declining in cultures derived from older postnatal animals (Delfs J, 1989). Because it is critical for the analysis of axonal growth that there is very good neuronal survival and a reliable formation of the central fiber tract we have decided not to use cultures from animals older than P6. Nevertheless, longitudinal spinal cord cultures from animals older than P6 might be appropriate for other studies, e.g. addressing neuronal survival.

4.5.2   Synaptic contacts and myelination in spinal cord slice cultures

In this study we have not analyzed electrophysiological activity within the cultures. Immunohistochemical staining with synaptophysin, a presynaptic marker, showed extensive and intense labelling throughout the culture indicating the formation of synapses. As there are several studies addressing electrophysiological activity in transverse slice cultures (Ballerini L, 1998; Tscherter A, 2001; Lu VB, 2006) it can be assumed that there will be the generation of a functional network also in the longitudinal cultures. The specific location of synaptophysin immunoreactivity along the dendrites of SMI32-positive motoneuron like cells as shown in Fig. 4.5 clearly suggests the presence of synaptic contacts.

In addition we have observed myelin ensheathment of longitudinally extending axons in the cultures. Cultures were analyzed after different time points in vitro and it was possible to

observe several stages of myelin formation within the cultures. At early time points, many oligodendrocytes were present in the process of early ensheathment of axons (Fig. 4.6 A, B) in the typical manner that one oligodendrocyte has started to enwrap multiple axons. At later time points there was extensive myelination of the axons present in the culture (Fig. 4.6 C, D). This shows that all stages of myelination proceed within the cultured slice. The time course of myelination in the cultures is comparable to the onset and sequence of myelination in vivo (Schwab ME, 1989). This slice culture model is thus suited for studies of myelin formation in the spinal cord, and provides an in vitro model system with the presence of myelinated axons.

4.5.3 Spontaneous axonal regeneration in spinal cord slice cultures

In our experiments we have observed spontaneous growth of spinal cord axons across a mechanical lesion introduced in the cultures. In agreement with previous studies (Prang P, 2001; Li D, 1995; Mingorance A, 2006) we did also observe a decline in the number of fibers crossing the lesion with increasing maturation in culture. This decline goes together with the emergence of myelin (see Fig. 4.6) and myelin-associated inhibitory molecules (Schwab ME, 1989; Schwab, 2004) in the spinal cord cultures and probably with a decline in the intrinsic capacity for neurite outgrowth of the spinal cord neurons (Zhou FQ, 2006). Nevertheless, even in the most mature cultures (lesioned at DIV14) there still was a substantial number of fibers which did cross the lesion site (Fig. 4.8F, Fig 4.9). Similarly, also in cultures derived from P6 spinal cord there still was a considerable number of axons crossing the lesion site after lesions made at DIV4. This indicates that, despite a general decline in the ability to regenerate, there is a subpopulation of fibers even in mature cultures which appears to retain this ability. Rather little is known about the potential of intrinsic spinal cord fibers for regeneration in vivo. Most in vivo studies have addressed either fiber populations descending from the brain or ascending from the dorsal root ganglia (for review see Kwon BK, 2002). However, growth of local spinal cord axons into and across a spinal cord lesion or into guidance channels has been described in some cases (Ma M, 2004; Xu XM, 1999). Recently there has been increasing recognition that besides the regrowth of the descending fibers from the brain sprouting of local fibers and of spinal cord connections is likely to contribute significantly to functional recovery after spinal cord injury (Bareyre FM, 2004; Bradbury EJ, 2006; Priestley, 2007). Our finding of a limited

spontaneous capacity for regrowth of intrinsic spinal cord axons thus is of considerable relevance for research on spinal cord injury. In a first experimental study we have been able to show that this spontaneous growth capacity can be stimulated further by pharmacological treatment with the compound rolipram which is known to improve axonal regeneration by elevating cyclic AMP levels (Nikulina E, 2004). This demonstrates that this culture model can serve as a tool for evaluating compounds known or thought to improve axonal regeneration in a spinal cord environment without initially having to do in vivo animal experiments when assessing a new compound.

5. *S*PINAL *C*ORD *C*O-*C*ULTURES:

Axonal growth in spinal cord organotypic slice co-cultures

The following section is unpublished results.

5.1   Introduction

It was previously shown that in an organotypic slice culture model, spinal cord intrinsic axons have the ability to grow across a lesion. This ability decreases with increasing maturation of the tissue. In these cultures axons become myelinated and form synaptic contacts. Moreover, pharmacological treatment with Rolipram, promoted the growth of regenerating fibers through a lesion site (Bonnici B, 2008). Unfortunately, with the spinal cord slice culture it is not always possible to detect improvements in growth through the lesion after treatment (observation from own results). The reason is that even in mature cultures, there is still a considerable number of fibers growing across the lesion which makes it difficult to detect changes. Taking older cultures is not feasible because cell survival and overall quality of the slice cultures decline rapidly if the cultures are derived from mice older than P6. In order to improve the detection of fiber regeneration, we have developed a spinal cord – cerebellar co-culture model. This model allows us to analyze axonal elongation of spinal cord intrinsic axons into the cerebellar slice. We could show that spinal cord axons do enter the cerebellar slice in particular when early postnatal spinal cord is combined with postnatal cerebellum. With increasing age of the spinal cord there was reduced axonal ingrowth from the spinal cord to the cerebellum.

In a second set of experiments we have analyzed the potential of cortical axons to grow into spinal cord. This arrangement would simulate one of the classical lesion paradigms used in spinal cord repair, the regeneration of the corticospinal tract. In entorhino-hippocampal co-cultures 'young' EC has been shown to grow into 'old' hippocampus (Li D, 1995). Furthermore, in in-vivo studies, 'young' embryonic tissue has been shown to be capable of axonal growth both in hippocampus (Zhou CF, 1989) and in the spinal cord (Wictorin K, 1990). This could be an alternative approach in promoting repair of spinal cord injury.

5.2 Materials and Methods

5.2.1 Spinal cord – Cerebellar slice cultures

Spinal cord organotypic slice cultures were prepared from P1 – 2 mice as described in Section 4.3.1. Cerebellum was than dissected from P8 – 10 mice out and sliced with a tissue chopper (also 350µm slices). The sagittal cerebellar slice was then placed adjacent to the spinal cord longitudinal slice on a semipermeable membrane (Fig. 5.1 A). Cultures were incubated in incubation medium (50% MEM, 25% basal medium eagle, 25% heat inactivated normal horse serum, 0.65% glucose and 2mM glutamax; pH 7.3) at 37°C in humified air with 5% CO_2. The incubation medium was changed every second day. The cultures were then treated with the PKC inhibitors, GF209103X and Gö6976, and with the cAMP activators, rolipram and sp. cAMP, discussed in Chapter 3. The treatment started on the day of culture and continued until the cultures were fixed, after around 7 days in culture.
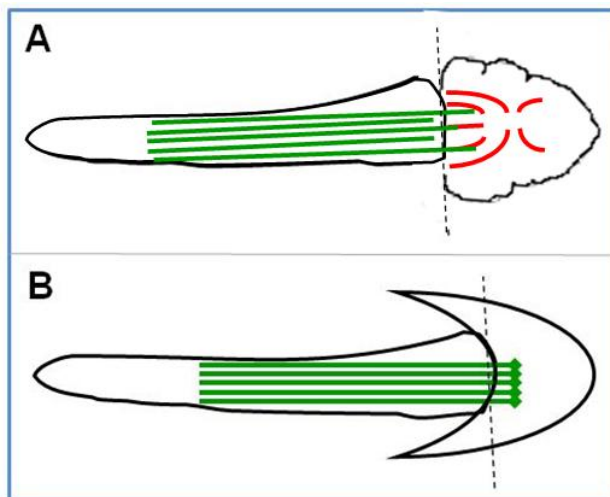
Fig 5.1 A. Spinal cord – cerebellar slice cultures and B. Cortex – Spinal cord cultures

5.2.2 Cortex – Spinal cord slice cultures

Spinal cord organotypic slice cultures were prepared from P1 – 5 rats as described in Section 4.3.1. Cortex was than dissected from P1 – 3 mice out and sliced with a tissue chopper (also 350µm slices). The cortical slice was then placed adjacent to the spinal cord longitudinal slice on a semipermeable membrane (Fig. 5.1 B). Cultures were then incubated in incubation medium for around 7 days.

5.2.3 Immunohistochemistry and microscopy

Cultures were fixed with 4% paraformaldehyde (PFA) for 2 h at room temperature (RT). For immunohistochemical staining cultures were washed 3 times for 10 minutes with phosphate buffered saline (PBS).

Spinal cord – cerebellar cultures were then blocked with a solution containing 0.5% Triton X-100 and 3% normal goat serum (NGS) for 20 minutes. This was followed by mouse anti-neurofilament SMI31 (Covance, Princeton NJ, USA; 1: 1000), and rabbit anti-Calbindin (1: 1000) as primary antibodies. Antibodies were diluted in PBS and applied either for 2 h at room temperature or overnight at 4 °C. Sections were then washed 3 times for 10 min with PBS and incubated with the secondary antibodies goat anti-mouse Alexa 488 (Molecular Probes; 1: 500), goat anti-rabbit Alexa 546 (Molecular Probes; 1: 500) for 2 h at RT.

In a slightly different staining procedure, cortex – spinal cord cultures were blocked with 0.1% TWEEN and 3% NGS for 20 minutes; followed by the primary antibody, rat anti-M6 supernatant (1: 50) in PBS at 4°C overnight. Cultures were washed for 3 times in PBS, and incubated with the secondary antibody goat anti-rat Alexa 488 for 2h at RT.

Sections were again washed twice with PBS, and mounted with Mowiol. Cultures were analyzed using an Olympus AX-70 fluorescence microscope and pictures were taken using a Spot digital camera. The brightness and contrast of the pictures was adjusted using Photoshop image processing software.

Using the 20x objective, the amount of fiber growth from the spinal cord to the cerebellum was quantified using the following a score system. The scores were obtained from at least 20 slices from 3 independent experiments. The statistical significance was assessed by Mann-Whitney's non-parametric test using Graph Pad Prism software.

5.3 Results

5.3.1 Spinal cord – Cerebellar slice cultures

In order to be able to differentiate axons coming from the spinal cord from intrinsic cerebellar fibers, the co-cultures were stained with SMI31 (green, Fig. 5.2) and Calbindin (red). SMI 31 stains neurofilaments of fibers from both spinal cord and cerebellum, while Calbindin is specific for cerebellar Purkinje cell processes. Since Purkinje cell axons are positive for SMI31 and calbindin while SMI31-poasitive spinal cord fibers are negative for Calbindin, it is possible to identify fibers originating in the spinal cord by being only SMI 31 positive (green, indicated by arrowheads in Fig. 5.2; while the fibers positive for SMI 31 and Calbindin are of cerebellar origin (orange, indicated by small arrows in Fig. 5.2) belong to the cerebellum.
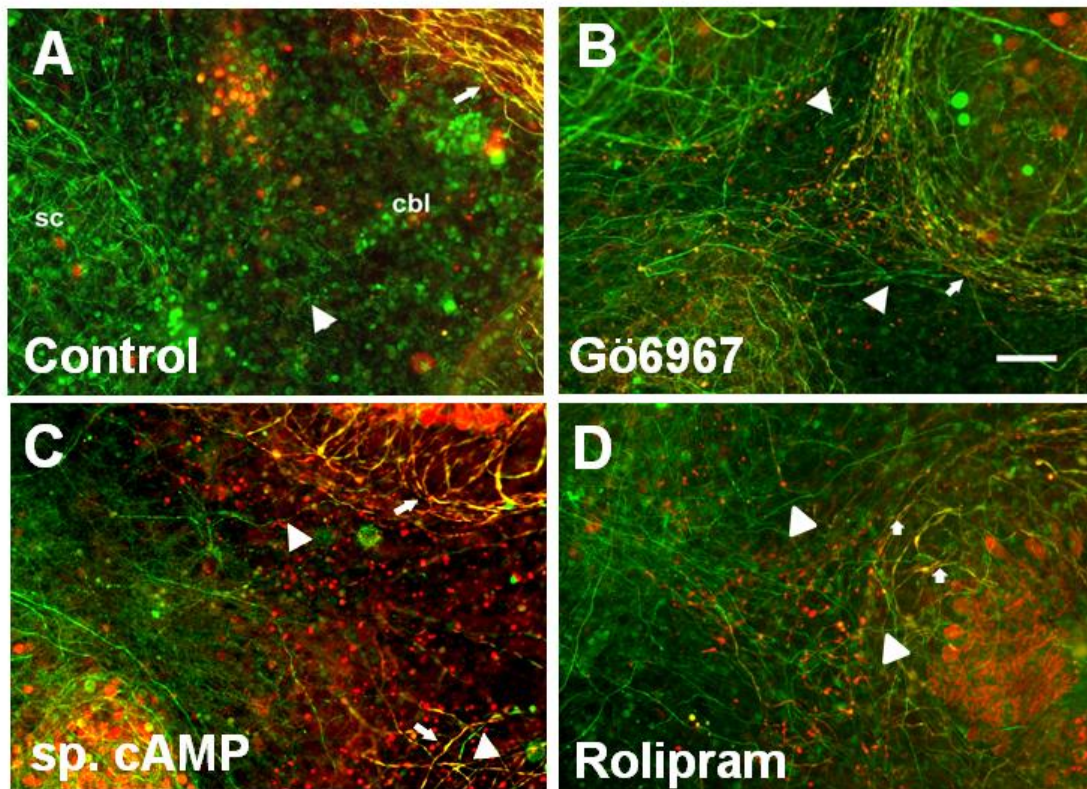
Fig. 5.2 Pharmacological treatments improve axonal growth from the spinal cord to the cerebellum.
A and C: in untreated cultures and cultures treated with sp. cAMP, there is no growth or only few fibers grow from spinal cord to cerebellum. B and D: when treating the cultures with the PKC inhibitor, Gö6967 (or GF109203X – not shown here) or with the cAMP activator, Rolipram, there is significant growth from the spinal cord to the cerebellum.

a. Untreated controls

To be able to see the effect of the treatments on the cultures, P1-2 spinal cord was co-cultured with P8-10 cerebellum. In this combination there is only little or no ingrowth of the spinal cord fibers into the cerebellum (Fig 5.3 A). For most of these controls, a score of 0 or 1 was given, indicating that there was no (score 0) or very few (score 1) fiber growth from the spinal cord to the cerebellum. (see Fig. 5.2 A and C for picture and Fig. 5.3 for score distribution).
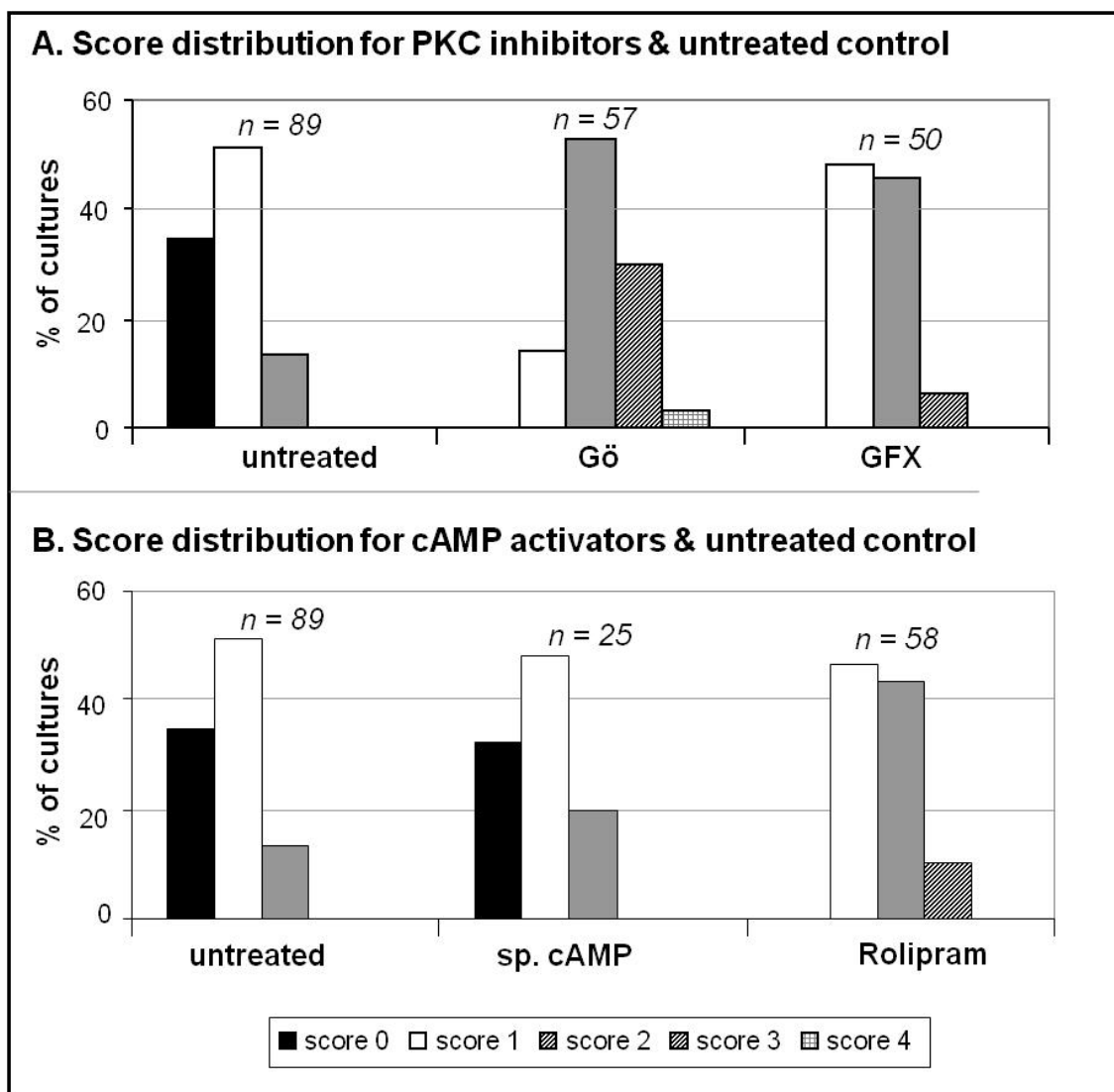
Fig. 5.3 Histogram showing score distribution for regenerating fibers of PKC inhibitors and cAMP activators. A. Cultures treated with PKC inhibitors show a significant increase in axonal growth when compared to untreated controls. B. with the cAMP activator Rolipram, there is also a significant increase in axonal growth, while sp. cAMP treatment does not show any increase in growth when compared to controls.

b. Treatment with protein kinase C inhibitors

The protein kinase inhibitors Gö6976 and GF109203X were tested for their potential to induce growth from the spinal cord to the cerebellum. In these treated cultures, growth was always observed and the scores ranged from 1, i.e., some growth to 4, i.e., strong growth as in fig. 5.2 B. With both treatments there was a large number of fibers crossing from the spinal cord to the cerebellum, showing statistically significant increase in number of regenerating fibers. These results are similar to previous results obtained both from our lab (Chapter 3; Prang P, 2001) and others (Sivasankaran R, 2004; Hasegawa Y, 2004).

c. cAMP Activators

The cAMP activators Rolipram and sp. cAMP were used to induce axonal growth. Although both expected to increase cAMP levels and thus promote axonal growth, for the cultures treated with Rolipram (*n=58)*, there was a significant increase in growth, while those treated with sp. cAMP (*n= 25)* did not show any significant increase ($P < 0.05$; *ns*) in the number of regenerating fibers (see Figs. 5.2 C, D). These results are comparable to the results we obtained previously in the model of hippocampal lesion (see Chapter 3). Fig. 5.3 B shows the distribution if these scores.

5.3.2   Cortex – Spinal cord slice cultures

In order to be able to distinguish the fibers growing from the cortex to the spinal cord form the spinal cords' own fibers, interspecies co-cultures were used with mouse cortex and rat spinal cord. The culture was than stained for M6, which is specific for mouse axonal projections, but does not label rat axons (Li D, 1993). Long projections grew from the cortex to the spinal cord, as shown in Fig. 5.4.
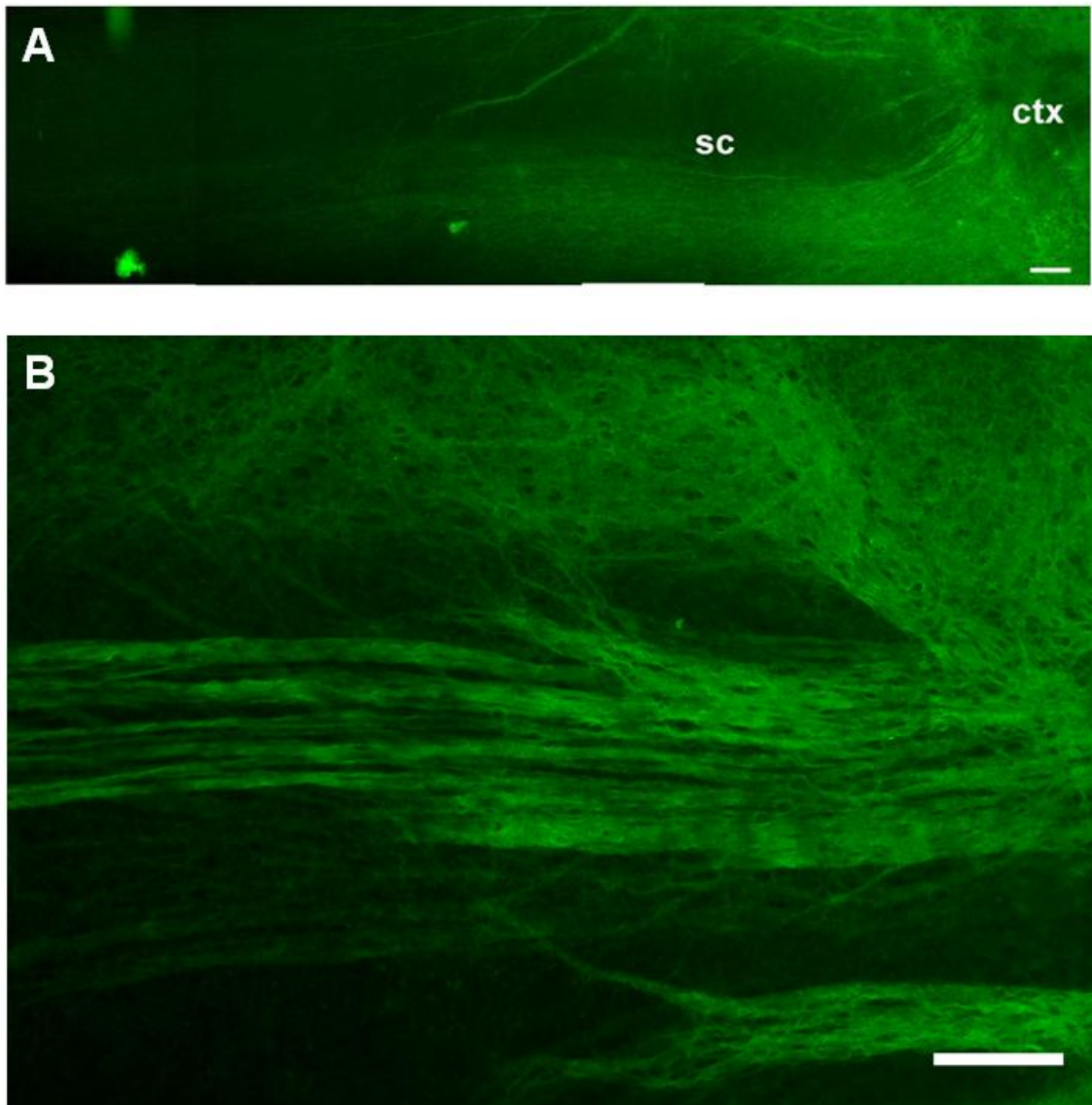
Fig. 5.4 Long axonal projections grow from the cortex to the spinal cord cultures.
A. Composite picture showing axons from mouse cortex stained with M6 projecting into rat spinal cord. and B. high power picture from another slice showing individual fiber projections from the cortex to the spinal cord.   Scale bar: 100µm

5.4   Discussion

In this study, we further developed the spinal cord slice culture model and were able to study the growth of spinal cord axons into cerebellar tissue. We could show that spinal cord axons do enter the cerebellar slice in particular when early postnatal spinal cord is combined with postnatal cerebellum. Various in-vivo tracing studies show that in the cervical spinal cord, the

central cervical nucleus and neurons in laminae VI - VIII give rise to spinocerebellar projections (Matsushita M, 1979). In this project we have not determined whether the axons growing into the cerebellum correspond to spinocerebellar fibers or come from other fiber projections entering the cerebellum aberrantly.

With increasing age of the spinal cord there was reduced axonal ingrowth from the spinal cord to the cerebellum (observation from own results). We then went further to try to improve this growth by treating these cultures with PKC inhibitors and cAMP activators. PKC is known to inhibit axonal growth by activating the Rho pathway. Various studies have shown that treatments with PKC inhibitors promote axonal growth both in-vitro and in-vivo (Prang P, 2001; Sivasankaran R, 2004; Hasegawa Y, 2004). On the other hand, cAMP activators are known to promote axonal regeneration; thus increasing the cAMP levels pharmacologically should induce axonal regeneration. Various studies have shown that treatment with both with cAMP alone, or with Rolipram (which also increases cAMP levels) induces axonal growth (Filbin, 2003; Spencer T, 2004; Nikulina E, 2004). In an entorhino-hippocampal lesion slice culture model from our lab (see Chapter 3) treatment with rolipram induced axonal regeneration, but treatment with sp. cAMP did not. The growth promoting affects of PKC inhibitors and cAMP activators from the spinal cord to the cerebellum were similar to our previous model of entorhino-hippocampal (see Chapter 3).

In addition, we also show early postnatal cortex is able to grow robust long axonal projections into the spinal cord. In a similar study, it was shown that neural progenitor cells promote corticospinal axon growth in similar co-cultures (Kamei N, 2004). Regeneration of corticospinal fibers has also been studied in in-vivo experiments. It was shown that treatment with neurotrophic factors, including BDNF and GEDNF (Zhou L, 2003) or treatment with the anti-NOGO antibody, IN-1 (Thallmair M, 1998; Z'Graggen WJ, 1998) significantly increase axonal sprouting and recovery of behavioural performance (IN-1 treatment). It would be interesting to investigate further whether this co-culture model is able to yield better axonal growth when combined with pharmacological treatments.

These results demonstrate further that organotypic slice cultures can be a useful tool for the study of axonal growth and regeneration within the spinal cord environment. Intrinsic spinal

cord axons have a considerable potential for spontaneous regeneration in the early postnatal period and are able to grow not only through a mechanical lesion, but also into another tissue. Moreover, utilizing these cultures we have started to evaluate the ability of descending cortical axons to extend in the spinal cord cultures and of various compounds and pharmacological agents to promote axonal regeneration and repair within the spinal cord environment.

6. GENERAL DISCUSSION

6.1 Use of various organotypic slice culture models to study axonal growth and regeneration

Organotypic slice cultures have been used for several studies, including axonal growth and regeneration of CNS. So far, the organotypic slice culture model used most for studying axonal regeneration has been the entorhino-hippocampal slice culture. Slice cultures allow experimental manipulations aimed at improving the regeneration of the lesioned fibers e.g. by addition of antibodies and pharmacological agents to promote regeneration.

Spinal cord organotypic slice cultures cut in the transverse plane have been used for a long time (Crain SM, 1963). Although these cultures are very useful for morphological (Delfs J, 1989) and electrophysiological (Phelps PE, 1996) studies, axonal regeneration cannot be studied with such cultures. In the second part of this project, we developed a longitudinal spinal cord organotypic slice culture model (Bonnici B, 2008). In these cultures longitudinal sections of the cervical spinal cord are used to allow fibres to extend over several segments. Cultures from animals older than P6 were not used since the fibre tract did not develop consistently. Neuronal survival also decreased in spinal cord cultures derived from mice older than P6 (Delfs J, 1989). Immunohistochemical studies using the presynaptic marker, synaptophysin show the extensive presence of synapses. Various electrophysiological studies in transverse sections indicate that these synapses are functional (Lu VB, 2006). Staining for myelin basic protein (MBP) during various time points in culture showed an increase in myelination along the axonal projections with increasing time in culture.

Longitudinal spinal cord organotypic slice cultures were then used to assess the spontaneous growth of axons through a lesion. Spontaneous axonal growth decreased with increasing time in culture. These observations are similar to others made in enterohinohippocampal slice cultures (Li D, 1995; Prang P, 2001; Mingorance A, 2006). Moreover, when the lesioned cultures were treated with Rolipram (discussed later), an increase in axonal growth through the lesion was observed.

Several organotypic co-culture models systems have been used to assess axonal growth and repair in neuroscience research. In this project, spinal cord – cerebellar co-cultures were developed to study more accurately and the growth of spinal cord axons after pharmacological treatment. By staining the cultures with SMI 31 and Calbindin, we were able to distinguish the axons coming from the spinal cord only (stained with SMI 31) from the axons found in the cerebellum (stained with both SMI 31 and Calbindin). In other experiments, we looked at the potential of cortical axons to grow into spinal cord. By co-culturing rat spinal cord with mouse cortex, and staining with the mouse specific neuronal marker, M6 we were able to observe long projections of cortical axons grow into spinal cord. This may also be used as part of a treatment of SCI.

6.2 Modification of signal transduction pathways to promote axonal regeneration

Various approaches to promote axonal growth and regeneration have been performed, including growth of axonal projections from young EC into old hippocampus (Li D, 1993; Radojevic V, 2004); and use of genetic approach, such as overexpression of Bcl-2 or GAP-43 (Solé M, 2004). In addition, various pharmacological and biochemical treatment approaches have been used by various labs to try to enhance axonal growth. This includes treatments with neurotrophic and growth factors (Woodhams PL, 1996; Prang P, 2001); with the NgR blocker NEP1 – 40 (Mingorance A, 2006), and using various effectors of signal transduction mechanisms (Prang P, 2001; and own results).

In this thesis, we have looked at modification of various signal transduction pathways to promote axonal growth in 2 different kinds of organotypic slice cultures: (1) from the EC to the hippocampus through a lesion and (2) from spinal cord to cerebellum in a co-culture model. On treating these cultures with the PKC inhibitors, Gö6967 and GF29003X, there was an increase in fibre growth in both models. These results are similar to those observed with GF29003X treated hippocampal slice cultures (Prang P, 2001). In an in-vivo study, Gö6967 promoted axonal regeneration in a spinal cord injury model. These culture models were also treated with the cAMP activators, sp. cAMP and Rolipram. Elevation of cAMP in known to promote axonal growth. Several studies show that compounds elevating cAMP improve axonal regeneration in spinal cord injury (Qiu J, 2002; Gao Y, 2003; Nikulina E, 2004). This growth-promoting effect is similar to the results we obtained when treating these cultures with

rolipram. However, sp. cAMP treatment did not promote axonal regeneration. One of the reasons we think is that this compound did not diffuse enough to reach therapeutic doses within the thick organotypic slice culture.

In the hippocampal slice culture model, other inhibitors of signalling pathways were also tested. Similar to what was observed by others previously, treatment with Pertussis toxin promoted axonal regeneration in our cultures (Prang P, 2001). Another compound which promoted axonal regeneration in our cultures is the IP3-Receptor antagonist, Xestospongin. The IP3 pathway promotes the PKC inhibitory pathway by releasing intracellular Ca^{2+}. This was the first time this compound has been used successfully to promote axonal regeneration. When treating the cultures with the ROCK inhibitor, Y − 27632 and the PI3 Kinase blocker, LY294002; unexpected results were obtained. Y-27632 has been shown to promote axonal regeneration of spinal cord injury in-vivo (Dergham P, 2002; Fournier AE, 2003). In our cultures this did not occur. On the other hand, treatment with the PI3K inhibitor, LY294002 has promoted axonal regeneration in our cultures.

6.3 Organotypic slice cultures versus in vivo

In this project we developed a longitudinal spinal cord organotypic slice culture system, and have used entorhino-hippocampal slice cultures to assess axonal regeneration. Organotypic slice cultures offer many advantages, they are easy to prepare and retain the cytoarchitecture of the tissue without having to perform the experiment on the whole organism, thus pharmacological and biochemical studies can be made more easily and a wider range of compounds can be 'scanned'. Eventually the studies need to be reconfirmed in the 'whole animal', but fewer animals are than needed, thus less complications due to surgery resulting in less suffering of animals. In addition, the mode of action of drugs and be tested first without the need to worry about the delivery method.

7. CONCLUSIONS & OUTLOOK

This project shows that organotypic slice cultures are a useful tool to study axonal growth and regeneration in various CNS injury models. Moreover, slice cultures can be used to evaluate compounds for their potential to promote axonal regeneration and pharmacological modulation of signal transduction pathways. We have shown that some of these compounds improve axonal growth / regeneration in the enterohinohippocampal and spinal cord slice culture models. This can be a promising approach for promoting CNS axonal repair. The synergistic effect of a combination of these treatments has not been studied in this project. This could be another interesting approach, which might yield even better regeneration.

In the longitudinal spinal cord slice culture model that was developed in this project we have also seen extensive presence of synapses and myelin formation within the culture. This indicates that this model could be used also for studies of synapse formation, and of myelin formation and maintenance which would be relevant for demyelinating diseases.

We have also seen that cortex extends long fibre projections in the spinal cord. It might be possible to even combine this with the positive pharmacological treatments to further promote this growth.

Finally, the compounds which promote axonal regeneration in this study need to be followed up by in-vivo studies and later by clinical trials. It is good to keep in mind that while this method can is useful to 'scan' for compounds promising to promote axonal regeneration, various 'obstacles' need to be overcome, such as mode of delivery, and side effects, before these compounds can be used successfully to promote axonal regeneration.

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PhD thesis:	Axonal regeneration in hippocampal and spinal cord organotypic slice cultures.
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LANGUAGES

English	Excellent knowledge
Maltese	Excellent knowledge
Italian	Very good knowledge
German	Good knowledge
Portuguese	Basic knowledge

SCIENTIFIC SKILLS

General Skills:
- GMP & GLP in pharmaceutical industry
- Supervision of students & technicians
- Scientific writing: articles; study & travel grant applications
- Managing small scientific projects
- General IT skills – Word, Excel, PowerPoint, GraphPad Prism

Cell & Molecular Biology:
- Organotypic slice cultures, including working in sterile environment and pharmacological treatments
- Animal techniques, including dissection of brain and spinal cord from mice & rats, intracardiac perfusion, drug injection, blood and urine collection, animal husbandry
- Immunostaining & Microscopy - fluorescence and confocal microscopy and analysis
- Molecular Biology - RNA extraction, Real time PCR, Western Blot

Pharm. Tech. & Chemistry:
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PUBLICATIONS

Peer Reviewed Publications:
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- <u>Bonnici B</u>, Kapfhammer JP. *Spontaneous regeneration of propriospinal axons in a novel spinal cord slice culture model.* Eur J Neurosci. 2008 May;27(10):2483-92

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- <u>Bonnici B</u>, Kapfhammer JP. *Promoters of regeneration of enterohinal fibers in mouse hippocampal slice cultures.* Article in preparation. (Work presented in Göttingen Neurobiology Conference. Göttingen, 2007)

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