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Neurotroofilised faktorid ja laminiin põhjustavad sensoorse närvisüsteemi neuronite aksonite kasvu kahe erineva signaaliraja vahendusel

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Neurotrophic factors and laminin activate two signalling pathways that trigger axonal growth in sensory neurons

MASTER´S THESIS

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Table of contents

Abbreviations

ARTN – artemin

ATP – adenosine trisphosphate

BDNF – brain-derived neurotrophic factor

BMP – bone morphogenic protein

Cdc42 – cell division cycle 42

Cdk5 – cyclin dependent kinase 5

CGRP – calcitonin gene-related peptide

CNS – central nervous system

CNTF – ciliary neurotrophic factor

CSPG – chondroitin sulphate proteoglycan

DRG – dorsal root ganglion

ECM – extracellular matrix

Erk – extracelluar signal-regulated kinase (or mitogen-activated protein kinase)

FAK – focal adhesion kinase

GAG – glycosaminoglycan

GDNF – glial cell-line derived neurotrophic factor

GFLs – GDNF family ligands

GFP – green fluorescent protein

GFRα - GDNF family receptor type α

GSK3 – glycogen synthase kinase 3

IL-6 – interleukin 6

ILK – integrin linked kinase

LIF – leukaemia inhibitory factor

MEK - mitogen-activated protein kinase kinase (or extracelluar signal-regulated kinase kinase)

NCAM – neuronal cell adhesion molecule

NGF – nerve growth factor

NRTN - neurturin

NT-3 – neurotrophin 3

NTF – neurotrophic factor

p75NTR – 75 kDa neurotrophin receptor

PC12 – pheochromocytoma cell-line

PCR – polymerase chain reaction

PGP 9.5 – protein gene-product 9.5

PI3K – phosphatidylinositol-3-kinase

PSPN – persephin

RET – rearranged during transfection

RTK – receptor tyrosine kinase

SFK – Src family kinase

SrcDN – dominant negative form of Src

SU6656 - 2,3-Dihydro-N,N-dimethyl-2-oxo-3-[(4,5,6,7-tetrahydro-1H-indol-2-yl)methylene]-1H-indole-5-sulfonamide

Trk – tropomyocine receptor kinase

Introduction

Sensation makes up the part of a living organism that perceives its outer environment and internal condition. In vertebrates, spinal cord injury can rob the injured ones of part of their sensation that deals with mechanical and proprioceptive stimuli. Even though only a small proportion of humans suffers from paralysis caused by spinal cord injury, this state is mostly incurable.

Dorsal root ganglion (DRG) neurons are responsible for sensing temperature, touch, vibration and pain from the skin and proprioceptive information. Several growth-inhibiting molecules in the glial scar tissue and lesioned myelin block regeneration of their axons into the spinal cord. Also the intrinsic activity of adult DRG neurons does not support efficient regeneration. Therefore studying the mechanisms of axonal growth has become an important field of neuroscience.

Axonal growth involves complex cellular processes like cell polarization and cytoskeleton rearrangement. These processes are triggered by several intra- and extracellular signals. An axon protrudes on a permissive substratum towards growth promoting soluble molecules like neurotrophic factors. Nerve growth factor (NGF) and glial cell-line derived neurotrophic factor (GDNF) are two neurotrophic factors that are promising for potentiating axonal growth through the hostile environment of the glial scar after central nervous system injury. One of the most widely studied permissive substrates for axonal growth is laminin.

The molecular mechanisms behind axonal growth have been studied in several aspects both *in vitro* and *in vivo*. Several signalling pathways have been discovered that link some extra- or intracellular signal to axonal growth. However, there is very limited information available about signal integration inside the cells that meet more than one input at a time, which is the actual case in developing or regenerating organism.

The theoretical part of this thesis gives an overview on DRG neurons and their role in sensation, the importance of neurotrophic factors and extracellular matrix molecules in the development and regeneration of the nervous system. In the experimental part, the thesis describes experimental work done to temporally distinguish between two signalling inputs, neurotrophic factors and extracellular matrix, which lead to axonal growth in DRG neurons. The intracellular molecules that are activated by these inputs are analysed and determined. At the end of the work the author suggests a Boolean model for the interaction of these two pathways and describes an *in vitro* model for the glial scar.

All work, experimental and theoretical, presented in this thesis was carried out at the Institute of Biotechnology, University of Helsinki. Most of the results are included in the manuscript that is attached to the thesis as an appendix.

Literature review

1. Dorsal root ganglion neurons and their role in the sensory nervous system

The somatic sensory nervous system

Sensation is an organism's ability to detect various external and internal signals. These include visual and auditory information, registering the body's balance through proprioception and vestibular organs, detecting tastes, smells and chemical substances. The somatic sensory system is responsible for carrying mechanical, thermal, proprioceptive and noxious stimuli from various receptors in the skin, skeleto-muscular systems and joints to higher centres of the brain.

The somatic sensory system can be roughly divided into two: pathways that relay information about mechanic stimuli and those that forward information about pain and thermal sensation. These two systems can be discriminated by different pathways by which the signal reaches the final target: the somatosensory cortex (see Figure 1).

The mechanosensory/proprioceptive pathway starts with mechanical stimulus of mechano- or proprioceptors. The signal from the body (except from neck and face) is forwarded towards the central nervous system by large myelinated axons of DRG neurons into the spinal cord. The axons of DRG neurons synapse in the brain stem nuclei, from these the signal is carried on to the ventral posterior lateral nucleus of the thalamus and finally to the somatosensory cortex. This pathway therefore includes three major synapsing points and is called the dorsal column pathway (Purves et al., 2004).

Information about pain and temperature is conveyed by lightly myelinated or unmyelinated axons through the spinothalamic tract to the somatosensory cortex. The axons from DRG neurons make the first synapse in the ventral horn of the spinal cord. The axons of the second order neurons cross the midline and enter the anterolateral part of the grey matter of the spinal cord. The second order axons synapse to the third order

neurons in the ventral posterior nucleus of the thalamus which forward the signal towards the primary somatosensory cortex (Zigmond et al., 1999; Purves et al., 2004).

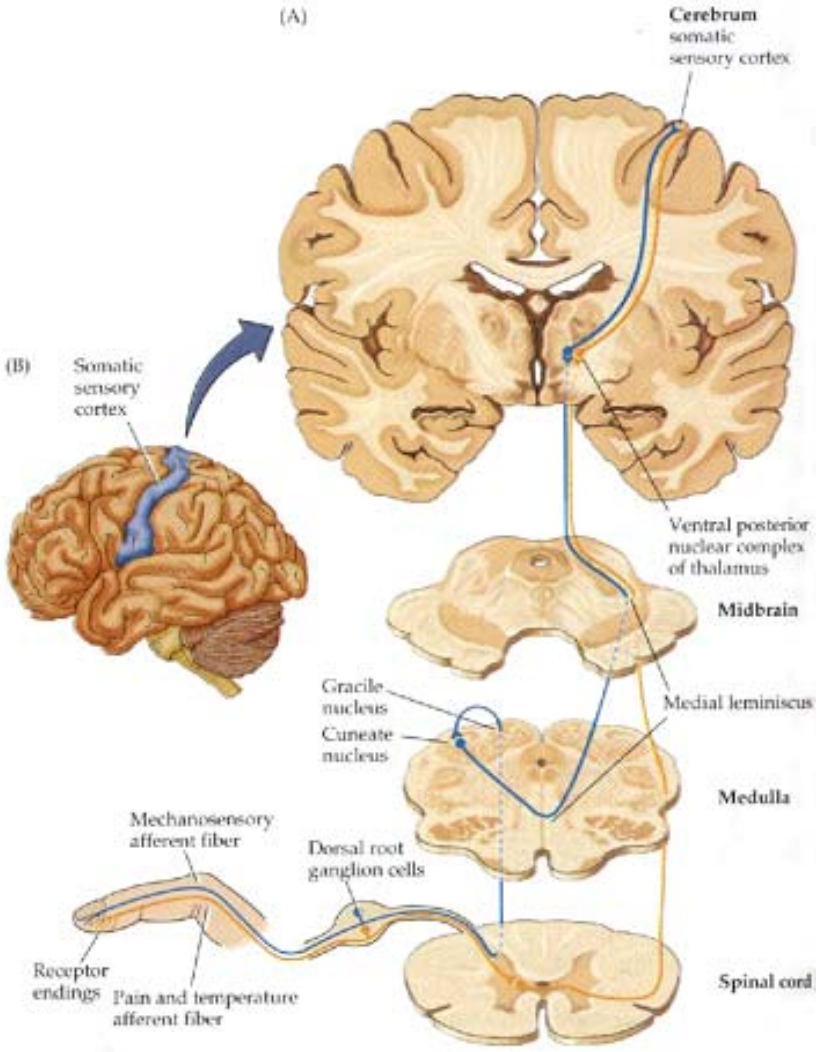


FIGURE 1. *Main pathways for the somatosensory information from the body (except from the head and neck). (A) The schematic pathway and synapses for mechanosensory information is depicted with blue line, for pain and temperature pathways with orange line. (B) The area of somatic sensory cortex is depicted in blue. (Purves et al., 2004)*

I would hereby draw the attention to the fact that the above-described pathways apply to transferring information from the body parts except from the head and neck. The first order neurons in the latter case are trigeminal ganglia neurons that forward the information towards different brain-stem centres.

Dorsal root ganglion neurons

As described in the previous section, DRG neurons are the first order neurons that direct sensory stimulus from the skin, tendon organs and skeletal muscle spindles to the central nervous system. They are aligned adjacent to the dorsal part on both sides of the spinal cord between each segment of the vertebral column.

Dorsal root ganglia and its neurons develop from a population of multipotent neural crest cells (Marmigere and Ernfors, 2007). The necessary factors that direct the neurons towards the sensory lineage are WNT1 and WNT3A that operate through activating transcription factors neurogenin 1 and 2 (Hari et al., 2002). Low levels of the bone morphogenic factor 2 (BMP2) also play an important role in the development of sensory neurons (Lo et al., 2002). Interestingly, high concentrations of BMP2 direct the neurons towards the autonomic lineage (Kleber et al., 2005).

The morphology of DRG neurons reveals very well their function. DRG neurons do not have any dendrites and they do not receive any input from other neurons. Their axons bifurcate close to the cell soma, one axon receiving signals from the periphery and the other one relaying this information towards the spinal cord. The region from which the DRG neurons receive their signal from different body segments is common between individuals. The skin of a human body is therefore divided into dermatomes *i.e.* a region of skin that is innervated by a specific DRG. The dermatome borders are not exact, but nevertheless this information helps to determine the level and location of nerve injuries (Zigmond et al., 1999; Purves et al., 2004).

There are several types of DRG neurons responsible for transferring different stimuli. The subtypes of DRG neurons can be distinguished by various parameters like the size of the cell soma, content of neuropeptides, expression of cell surface receptors and ion channels. For example, the neuropeptides CGRP (calcitonin gene-related peptide) and substance P are usually found from small diameter neurons (Gibson et al., 1984) that innervate skin and viscera while neurofilaments are abundant in large diameter cells

innervating muscle spindles (Gibson et al., 1984; Skofitsch and Jacobowitz, 1985). The expression of neurotrophin receptors during the development of DRG neurons has been well studied. It has been shown that small diameter neurons with thinly myelinated or completely unmyelinated axons express TrkA receptor and are responsible for nociception, thermoception and low-threshold mechanosensation. Large diameter cells with myelinated axons responsible for mechanosensation express TrkB and/or Trk C and proprioceptors express TrkC receptor (McMahon et al., 1994; Farinas et al., 2002). The expression patterns of different receptors also change in some sub-populations of DRG neurons. For instance, TrkA receptor is expressed by over 80% of embryonic neurons, while postnatally only by about 50% of neurons express this receptor. It has been shown that lectin IB-4 positive small neurons in mice switch from dependency of NGF to GDNF during postnatal life (Molliver et al., 1997). A more thorough overview of different neurotrophic factors and their receptors will follow in the next chapter.

Spinal cord injury

According to statistical studies, there are 15-40 traumatic spinal cord injury cases per one million of population, the higher numbers representing more industrialized countries like the USA as an example. Even though the frequency of the trauma is low, the outcome is very severe. Spinal cord injuries lead to significant and catastrophic dysfunction and disability of patients of whom only less than 1% can hope for complete neurologic recovery (Lim and Tow, 2007). The traumatic outcome affects mainly young adults: the average age of incidence is below 40 years and 77.8% of the patients are male. Traffic accidents account for 46.9% of the aetiology of these injuries, other most common causes are industrial accidents, falls, acts of violence and sporting activities (Spinal Cord Injury Information Network http://www.spinalcord.uab.edu/).

The paralysis of the patients suffering from spinal cod injury derives partly from the fact that injured sensory axons are unable to regenerate into the spinal cord and re-innervate their original targets in the central nervous system. This inability is caused by the

unfavorable environment created by the reactive astrocytes at the lesion site. Secretion of growth inhibitory molecules leads to the development of the glial scar, which is a type of tissue that adult axons are naturally unable to penetrate (Qiu et al., 2000).

Based on the important role of DRG in the development and trauma, it is important to study the axonal growth of sensory neurons, their response to several growth inhibiting and activating molecules. An overview of current knowledge and understanding of neurite outgrowth and the pathways regulating it are described in the following parts of this literature review.

2. Neurotrophic factors and their receptors

Diversity of neurotrophic factors

Neurotrophic factors were first characterized by their ability to promote the survival of neurons. In the 1940´s and 1950´s several experiments by Viktor Hamburger and Rita Levi-Montalcini showed the importance of programmed cell death in the development of the nervous system of chick embryo. These studies lead to the discovery and purification of the first neurotrophic factor NGF (nerve growth factor) by Stanley Cohen in 1960. The isolation and sequencing of the NGF protein followed in 1971 (reviewed in Levi-Montalcini, 1987 and Oppenheim, 1991). By today it has been shown that besides preventing cell death many neurotrophic factors may also perform other biological functions in the nervous system. They can support proliferation, neurite growth, migration, synapse formation and cell differentiation. Neurotrophic factors are therefore important molecules in the development, maintenance and plasticity of the nervous system. Textbooks usually divide neurotrophic factors into three larger subclasses: neurotrophins, GDNF family neurotrophic factors and neuropoietic cytokines (Zigmond et al., 1999). A more thorough overview will be given of the two first sub-classes.

The neurotrophin family

The neurotrophin family is the oldest and best characterized group of neurotrophic factors. As already mentioned above, NGF was the first of them to be purified and characterized. The other members of this group include BDNF (brain-derived neurotrophic factor) which was purified in 1982 (Barde et al., 1982), and two neurotrophins numbered 3 and 4/5 (NT3 and NT4/5 respectively).

Neurotrophins are synthesized in neuronal and non-neuronal cells as pre-pro-proteins. The signalling peptide is cleaved in the endoplasmatic reticulum and the proform is either further cleaved inside the cells (to form the mature protein) or it is secreted to the extracellular environment. Depending on the availability of certain proteases the proform can be cleaved also outside the cell (reviewed in Schweigreiter, 2006). Both, the mature and the proforms form homodimers (Kolbeck et al., 1994). These homodimers can bind to three different types of receptors: 1) Trk family of receptor tyrosine kinases, 2) p75 neurotrophin receptor (p75NTR) and 3) sortilin.

There are three types of Trk receptors identified in mammals. TrkA is a receptor for NGF, TrkB for BDNF and NT4/5, and TrkC for NT3. NT3 can also weakly bind to TrkA and TrkB. P75NTR belongs to the superfamily of tumor necrosis factor receptors and binds with similar affinity all four neurotrophins (Schweigreiter, 2006). Sortilin is the newest neurotrophin receptor purified by Petersen *et al* in 1997 (Petersen et al., 1997). It was first identified as a receptor for a neuropeptide called neurotensin (Mazella et al., 1998), but it has by now been shown to bind the proforms of NGF (Nykjaer et al., 2004) and BDNF (Teng et al., 2005). The best studied binding possibilities between neurotrophins and the three types of receptors are schematically described in Figure 2.

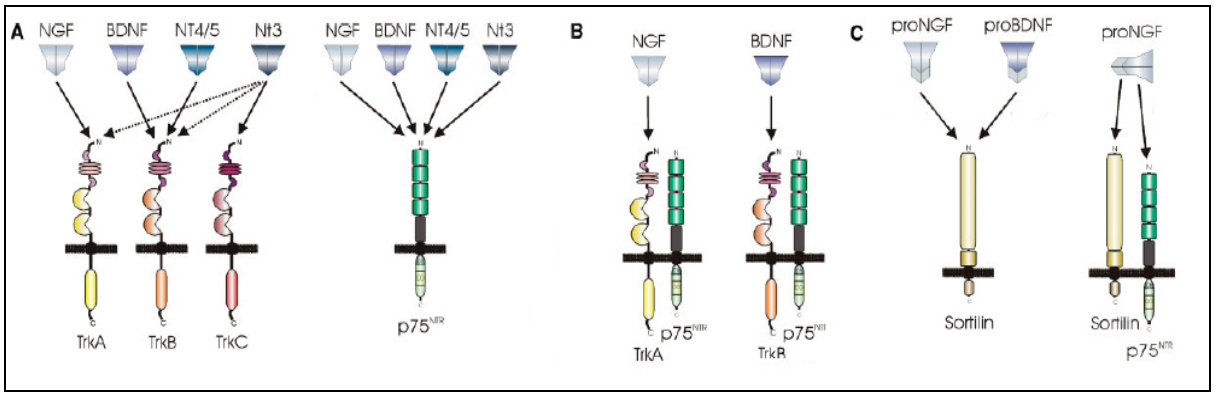


FIGURE 2. *Signalling of neurotrophins through their receptors: Trk, p75NTR and sortilin. (A) Specific binding of neurotrophins NGF, BDNF, NT4/5 and NT3 to their cognate Trk receptors. All four neurotrophins also bind to p75NTR. (B) NGF and BDNF have been shown to bind Trk-p75NTR receptor complex. (C) proNGF and proBDNF bind to sortilin, proNGF has been shown to bind to sortilin-p75 dimer. More detailed information of these interactions and their functions is described in the text. BDNF – brain-derived neurotrophic factor, NGF – nerve growth factor, NT – neurotrophin, p75NTR – p75 neurotrophin receptor. (The scheme is modified from Schweigreiter, 2006)*

The differences in the binding affinities between the neurotrophins and their receptors and the several possible combinations of receptors give a way for diverse signalling options and different biological outcomes. For example, neuronal survival is promoted by mature neurotrophins signalling via Trk receptors while pro-neurotrophins acting via p75NTR-sortilin complex trigger apoptosis. Apoptosis is triggered also through p75NTR receptor by mature neurotrophins, but a higher concentration of ligands is required for that activity. NT3 by binding to TrkC is responsible for proliferation and migration of Schwann cells during the embryonic development of the peripheral nervous system. On the other hand BDNF strongly inhibits the migration of these cells through p75NTR receptor and promotes cell differentiation (Schweigreiter, 2006). Neurotrophins and their receptors are also involved in long-term synaptic plasticity. There are several studies describing the role of NGF and BDNF in the enhancement of long-term potentiation (LTP) in hippocampal slices, a cellular analogue for learning and memory. According to these studies, neurotrophins act via Trk receptors. ProBDNF signalling through p75NTR was shown to facilitate the opposite, *i.e.* long-term depression. BDNF has also been shown to have neuroprotective effect and the defects of BDNF signalling might play a role in the development of Alzheimer's disease (Hennigan et al., 2007).

The GDNF family of neurotrophic factors

The GDNF family of neurotrophic factors (or GDNF family ligands or GFL-s) has been found to be critical in neurodevelopment, synaptic plasticity, neuronal survival, neurite outgrowth, but also in kidney development and spermatogenesis. This group of neurotrophic factors has four members. GDNF was first identified as a secreted protein from a glioma cell line that had survival promoting effects on midbrain dopaminergic neurons in culture (Lin et al., 1993). Three years later, neurturin (NRTN) was purified and shown to promote the survival of sympathetic and sensory neurons (Kotzbauer et al., 1996). The two last members artemin (ARTN) and persephin (PSPN) were found by homology-based PCR scanning (reviewed in Baloh et al., 2000; Airaksinen and Saarma, 2002; Saarma, 2003).

The classical model of signalling of the GFL-s includes RET receptor tyrosine kinase. GFL-s do not bind RET directly, but use co-receptors for signalling. GFRα1-4 (GDNF family receptor sub-types α) are the preferred RET co-receptors for GDNF, NRTN, ARTN and PSPN, respectively. These receptors are bound to the membrane by their GPI-anchor (glycosylphosphatidylinositol), but do not reach the cytoplasm like RET. Noticeable crosstalk between the ligands and the receptors exists *in vitro*: GDNF has also been shown to act via GFRα2, NRTN and ARTN can activate RET through GFRα1 (Baloh et al., 2000) as depicted in Figure 3. This classical signalling model has become more complicated after recent findings that suggest GDNF-GFRα1-RET complex signalling through heparan sulphate proteoglycans (Barnett et al., 2002) or that GFLs can signal independently of RET through neuronal adhesion molecule NCAM (reviewed in Sariola and Saarma, 2003).

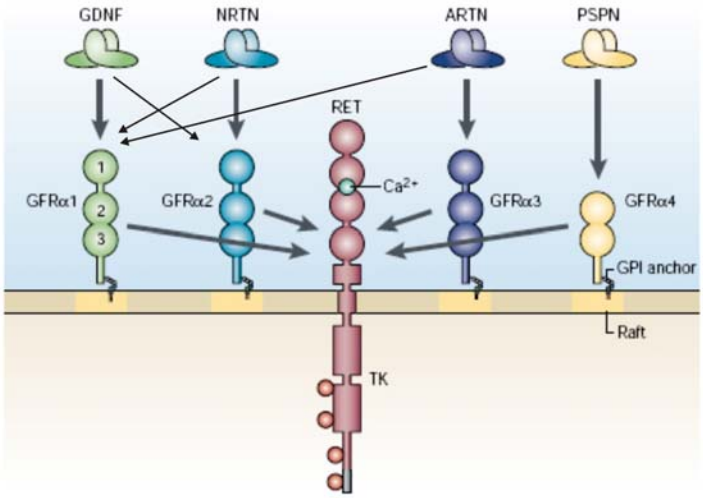


FIGURE 3. *The classical signalling scheme of GFL-s through GFRα and RET receptors. Each GFL has its specific GFRα co-receptor for binding RET receptor tyrosine kinase. Bold arrows refer to preferred bindings of ligands to their receptors. Thin arrows show the possible cross-talk between GFL-s and the GFRα receptors. ARTN – artemin, GDNF – glial cell-line derived neurotrophic factor, GFRα – GDNF family receptor type α, GPI – glycosylphosphatidylinositol, NRTN – neurturin, PSPN – persephin, TK – tyrosine kinase domain. (Scheme modified from Airaksinen and Saarma, 2002)*

By now, the effect of all four GFL-s on several types of neurons has been extensively studied. ARTN is required for the migration and initial axonal outgrowth in superior cervical ganglion sympathetic neurons signalling through its preferred receptors (Nishino et al., 1999). GDNF signalling is required during the embryonic development of parasympathetic neurons for migration and proliferation of precursor cells, and also for promoting migration, proliferation, survival and differentiation of precursor cells in the enteric nervous system (Taraviras et al., 1999; Enomoto et al., 2000; Rossi et al., 2000). The role of NRTN in the parasympathetic system is to maintain the innervation and neuronal size of the neurons and affect neurotransmitter release in enteric system in post-natal mammalian organism (reviewed in Airaksinen and Saarma, 2002). During embryonic development, GDNF and NRTN support the survival of motoneuron subpopulations, acting both through GFRα1 receptor (Garces et al., 2000; Oppenheim et al., 2000). In postnatal motoneurons GDNF promotes terminal axonal branching and synapse formation (Keller-Peck et al., 2001). GDNF and NRTN have been shown to be potential survival factors for midbrain dopaminergic neurons, GDNF being able to promote axon growth and hypertrophy of these neurons (Akerud et al., 1999).

In the sensory nervous system the embryonic development of DRG neurons is dependent on NGF, but postnatally a shift from NGF to GDNF dependence occurs (Molliver et al., 1997). Therefore neuronal survival is supported by GFL-s (GDNF, NRTN and ARTN) in postnatal but not in embryonic DRG cultures (Baudet et al., 2000). Studies on axonal growth have revealed that all GFL-s, except for PSPN, promote axonal initiation in DRG neuronal culture. GDNF and NRTN induce lamellipodial formation at cell somata and at the tips of growth cones and GDNF has a positive effect on axonal elongation (Paveliev et al., 2004).

Some of the most drastic biological activities of GFL-s and their receptors have been identified using knock-out mouse models. Mice that lack RET, GDNF or GFRα1 genes die at birth. They lack kidneys and have serious deficits in the development of the parasympathetic and enteric nervous systems. Also NRTN and GFRα2 knock-out mice show deficits in the parasympathetic and enteric nervous system innervation. The lack of GFRα2 and GFRα3 receptors leads to similar drooping eye-lid phenotype. This phenotype is produced by lack of innervation in lacrimal glands in GFRα2 and the lack of innervation in eye-lid elevator muscle in GFRα3 knock-out mice. PSPN has been shown *in vitro* to promote the survival of dopaminergic neurons in rats (reviewed in Airaksinen and Saarma, 2002; Baloh et al., 2000), and GFRα4 knock-out mice have impaired calcitonin secretion in the thyroid (Lindfors et al., 2006).

Due to the many biological effects described for GFL-s and their receptors, these molecules have received a lot of attention for their possible therapeutic value. Several studies have revealed a positive effect of GDNF and NRTN to Parkinson's patients and in animal models of Parkinson's disease due to their positive effect on the survival of dopaminergic neurons. Investigations of the sensory and motor systems have revealed a promoting effect of GDNF on axonal regeneration. Actively surveyed areas include also epilepsy, ischemic stroke and addiction (reviewed in Airaksinen and Saarma, 2002).

The neuropoietic cytokine family of neurotrophic factors

Members of this family of neurotrophic factors are structurally related cytokines: interleukin-6 (IL-6), IL-11, IL-27, leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin 1, neuropoietin and cardiotrophin-like cytokine. All these cytokines signal through the gp130 receptor using a factor-specific combination of co-receptors. In addition to their involvement in infection, pregnancy, and bone, muscle and cardiovascular function, these cytokines have also been shown to have signalling functions in the developing and adult brain, and in response to brain injury. LIF and CNTF have a role in switching between neurogenesis and gliogenesis during the development of the nervous system. LIF also regulates the expression of various neuropeptides, together with CNTF and IL-6 it can rescue motor neurons after axotomy, promote survival of oligodendrocytes in culture, and activate astrocytes and microglia in response to injury in the central nervous system. LIF and IL-6 are involved in mediating behavioural patterns, including those related to feeding, sleeping and stress (reviewed in Bauer et al., 2007).

3. The extracellular environment and cell adhesion

Neurotrophic factors alone are not sufficient to be responsible for such complex cellular activities as migration, axonal growth and guidance. There are also other molecules that provide the guidance cues for cell movement and cells have receptors to recognize these molecules. The so-called extracellular matrix (ECM) molecules can provide attractive or repulsive guidance cues and permissive or non-permissive substratum for the cells to carry out these complex biological activities (Zigmond et al, 1999).

Many classes of ECM molecules have been identified while studying basement membrane development in early embryos. The mammalian basement membranes consist primarily of laminins, collagens (mostly type IV collagens), nidogens, the heparan sulphate proteoglycan perlecan, fibronectin, tenascin-C and others. Laminins, type IV

collagen and nidogens have been shown to be present in all basement membranes in the organisms throughout the vertebrate and invertebrate phyla. Laminin and collagen IV have been found to separately form polymers while nidogen acts as an integrator of the basement membrane forming ternary complexes with the first two (Yurchenco et al., 2004).

Taking into account the information that would be relevant for the topic of this thesis, I am hereby concentrating more thoroughly on laminin, cellular receptors for laminin and repulsive guidance molecules, which have an important role during the injury of central nervous system.

Laminins and integrins

Each laminin molecule is a trimer consisting of one α, β and γ subunit (Figure 4). The variety of laminins differs according to the complexity of the structure of an organism. In *C. elegans* and *Drosophila*, two isoforms have been found, varying the α subunit (Huang et al., 2003). In more complex vertebrates five α, four β and four γ subunits have been characterized and at least 15 different laminins inferred (Yurchenco et al., 2004). Laminin, besides being absolutely necessary for the basement membrane construction and the development of various organs, is also a permissive substratum for axonal growth *in vitro* for several neuronal types, including DRG neurons (Dontchev and Letourneau, 2003; Turney and Bridgman, 2005; Tucker et al., 2006). Laminin subtypes 1 and 2 are expressed in various tissues of the developing central and peripheral nervous system and they probably have a positive role for the migration of neuronal stem cells and axon pathfinding (Colognato and Yurchenco, 2000).

The known receptors and receptor-like molecules for laminins are integrins, dystroglycans, sulphated carbohydrates, Lu blood group glycoprotein sulphates and other cell surface molecules. Only integrins and dystroglycan of these above-mentioned receptors are transmembrane molecules that bind and signal to the cell cytoskeleton

(Colognato and Yurchenco, 2000; Yurchenco and Wadsworth, 2004). The major receptor complexes that integrate signals from the extracellular matrix to axons are integrins. Integrins are homodimeric receptors, comprising of one α and one β subunit. At least 18 α and 8 β subunits have been found and 24 different combinations identified (Hynes, 2002). The β-1 family of integrins was identified as a mediator of neurite outgrowth for DRG neurons in response to extracellular matrix molecules combining with several subclasses of α subunits, for example α-1 or α-3 (Tomaselli et al., 1993).

Several genetic studies have revealed the function, localization and diversity of laminins and its receptors. Ongoing studies are being carried out on the matter of how laminins and its receptors mechanistically regulate development. A model of self-assembly of laminins has been proposed. This model predicts that cell-surface receptors anchor laminin and create localized concentration gradients of laminins. When the concentration exceeds a certain threshold, other components of basement membranes assemble. Polymerization further triggers the reorganization of cell surface receptors, which leads to the reorganization of cytoskeleton network inside the cell. In this way the cell and the extracellular medium are intermingled in constant dynamical events that lead to different biological outcomes (Colognato and Yurchenco, 2000; Huang et al., 2003).

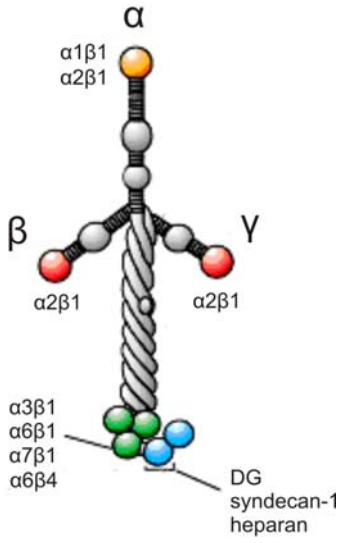


Figure 4. *A schematic structure of a laminin molecule consisting of an α, β and γ chain. Sites for binding different receptor molecules have been shown. αβ - integrin dimers, DG – dystroglycan. (Modified from Yurchenco and Wadsworth, 2004)*

Repulsive molecules of the extracellular matrix, the glial scar and myelin

It has been well known that there is no regeneration of the mature axons into the central nervous system while there are no restrictions to the regeneration of the peripheral nervous system. It has also been accepted that this difference does not derive from the axon's inability to regenerate, but from the non-permissive environment that the growth cone encounters when approaching the central nervous system (Qiu et al., 2000). In that respect, several studies on inhibitory guidance cues will be described in the following paragraphs.

Proteoglycans are made up of a protein core with one or more linear glycosaminoglycan (GAG) chains. These chains consist of repeating disaccharide units. Among several types of proteoglycans, chondroitin sulphate proteoglycans (CSPG) are the most abundant in mammalian central nervous system, where they act mainly as the barrier molecules affecting axonal growth, cell migration and plasticity (Laabs et al., 2005).

A glial scar develops at the site of the lesion in response to the central nervous system injury. It consists of proliferating and migrating astrocytes, microglia and oligodendrocytes that secrete several ECM molecules like CSPGs. Several regenerating axons stop when encountering the tissue of glial scars *in vivo* (Silver and Miller, 2004; Laabs et al., 2005) and specifically CSPG-s *in vitro* (Zhou et al., 2006). The mechanism of the inhibitory action of CSPG-s is not well understood. It has been shown that the GAG-domains of the molecule can bind several growth factors and cell adhesion molecules, and might have a role in sequestering them away from their receptors (Bovolenta and Fernaud-Espinosa, 2000). CSPG-s also bind to ECM molecules, including laminin, and can affect axon growth through blocking the growth promoting activity of laminin (Laabs et al., 2005).

The ECM molecules produced by reactive astrocytes are still not the only factors behind inefficient axon regeneration into the central nervous system. The myelin-associated inhibitors like Nogo, MAG (myelin-associated glycoprotein) and Omgp (oligodendrocyte

myelin glycoprotein) have been shown to have a role in this phenomenon. Also the cellular intrinsic activities of adult neurons compared to embryonic ones are proposed to be at least in part responsible for the inability to regenerate into the tissues of the central nervous system. It has been shown that on suitable conditions embryonic axons, unlike the adult ones, are able to grow into the CNS (Qiu et al., 2000; Filbin, 2003; Silver and Miller, 2004; Laabs et al., 2005).

4. Intracellular signalling pathways for axonal growth

Axonal growth is a complex and highly coordinated process that involves integrating signals from the extracellular space and reassembling cytoskeleton and cell membrane for growing protrusions. The growth gone of the developing or regenerating axon meets signals that are then transported via several molecules to the nucleus, where the expression of necessary genes takes place. Synthesis of new proteins takes place in the cell cytoplasm and these have to be transported back to the elongating axons and dynamic growth cones.

Signalling pathways activated by neurotrophic factors and laminins

The signals from the extracellular medium are transferred into the cell by various receptors. The receptors of neurotrophins and GFL-s (Trk and RET receptors respectively) described above belong to a group of receptor tyrosine kinases (RTK-s). Upon ligand binding the RTK receptor dimerizes and gets activated through autophosphorylation (reviewed in Schweigreiter, 2006 and Bespalov and Saarma, 2007). Several *in vitro* studies have established that Raf-Erk (extracellular signal-regulated protein kinase) and PI3K (phosphatidyl-inositol-3-kinase) pathways are necessary for neurotrophic factor-dependent neurite outgrowth and activated by RTK-s (reviewed in Zhou and Snider, 2006).

PI3K has been shown to regulate endocytosis of RTK-s and this step is crucial for intracellular transduction of signals from neurotrophic factors. PI3K pathway, in particular, is involved in several local events that control axon morphogenesis and are dependent on neurotrophic factors. These events include also branch formation (Gallo and Letourneau, 1998) and axon turning (Ming et al., 1999). PI3K has been shown to co-localize with polymerizing actin filaments at the leading edge of the growth cone. More specifically, PI3K-Cdc42 pathway has been shown to promote filopodia formation and axon elongation. PI3K-Akt signalling regulates actin polymerisation, cell motility and survival. PI3K-Rac pathway has been studied in migrating non-neuronal cells, where the signalling induces the formation of lamellipodia. If the same is also true for neuronal cells, is currently being disputed about. The activity of glycogen synthase kinase 3 (GSK3), a protein regulated by various branches of PI3K pathway, is involved in microtubule assembly during axon extension (reviewed in Zhou and Snider, 2006).

Erk-s are the major signalling mediators necessary for axonal growth and they are also activated by NTF receptors. In addition to their role in gene expression in the cell nucleus, Erk-s are also necessary for local axon assembly. Several studies in this field suggest that the main function of Erk in axons is to maintain the level of proteins necessary for responding to cues from the extracellular environment through regulation of protein synthesis and degradation. The proteins regulated by Erk and the local synthesis might include several molecules from raw materials like actin filaments to receptors and signalling mediators of the guidance cues. Gene expression in response to the signalling from neurotrophic factors is also under the control of Erk. The best described transcription factors downstream of Erk are CREB (cyclic AMP response element binding protein) and the NFATc (the nuclear factor of activated T-cells) family transcription factors. The signalling pathway upstream of Erk includes Ras-Raf-MEK signalling and can also be modulated by PI3K pathways through Rac and Cdc42 (cell division cycle 42) (reviewed in Zhou and Snider, 2006).

Laminins have been shown to have a potentiating effect on axonal growth induced by neurotrophic factors during the embryonic development. Importantly, neurotrophic

factors that promote axon growth, like NGF, have been found to cluster integrins at the leading edge of the growth cone (Grabham and Goldberg, 1996). The link between integrins and actin filaments has not yet been completely clarified. Actin motor proteins myosin II and myosin X have been granted at least part of this role. Integrins can also recruit neurotrophic factor receptors into various microdomains, like lipid rafts, affecting the signalling of growth factors. The result of such cross-talk is to increase the efficiency of signalling. The co-activation of integrins and receptors for neurotrophic factors is necessary for the full activation of focal adhesion kinase (FAK). This activation is again necessary for neurite outgrowth in PC12 cell-line (Ivankovic-Dikic et al., 2000). Finally, the inhibitory molecules of the glial scar CSPG-s have been shown to inhibit axonal outgrowth via interfering with integrin signalling. Over-expression of integrins has been shown to promote axon growth over CSPG-s (reviewed in Zhou and Snider, 2006).

Integrin signalling interacts with RTK signalling in various points (see a simplified scheme in Figure 5). One of the key players in this signalling cross-talk is integrin linked kinase (ILK). ILK is placed in the signalling pathway downstream of PI3K and binds to integrin β subunit. This kinase has been shown to modulate microtubule dynamics through inactivating GSK-3, regulating actin assembly and disassembly through Rac-Cdc42 pathway and interfering with Raf-MEK-Erk pathway (reviewed in Zhou and Snider, 2006). The same pathway is also regulated upon activation of FAK via Src kinase (Mitra et al., 2005).

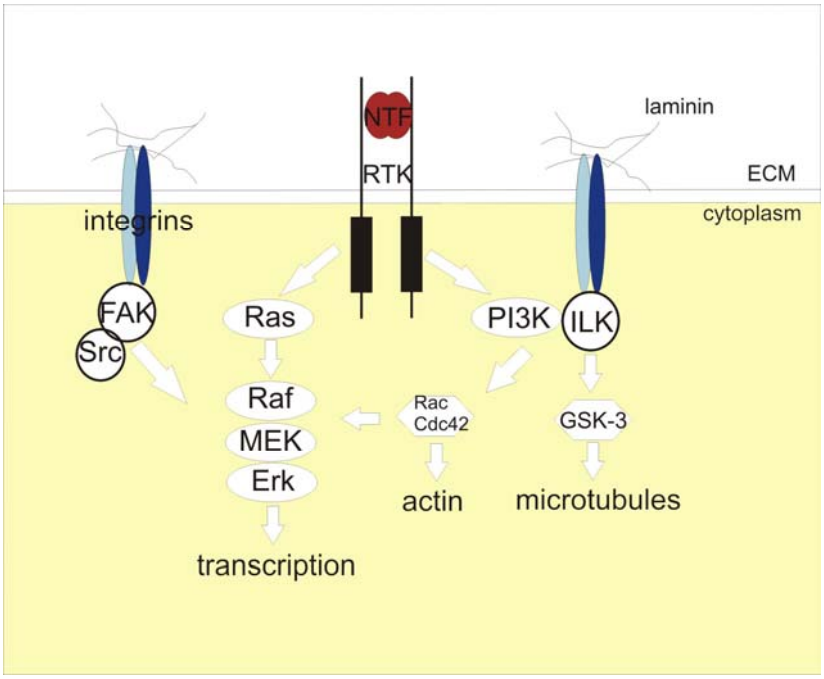


FIGURE 5. *A simplified schematic representation of signal integration from integrin and growth factor receptors. The inputs to this system are extracellular matrix (ECM) molecules binding to integrins and neurotrophic factors (NTF-s) activating receptor tyrosine kinases (RTK-s). Major results from these pathways include transcription and rearrangement of cellular cytoskeleton, including actin and microtubules in the growth cone for its progression or repulsion. Cdc42 – cell division cycle 42, Erk – extracellular signal-regulated kinase, FAK – focal adhesion kinase, GSK-3 – glycogen synthase kinase 3, ILK – integrin linked kinase, PI3K – phosphatidylinositol-3-kinase (after Zhou and Snider, 2006).*

The role of Src family kinases in axonal growth

The Src family consists of non-receptor type protein tyrosine kinases. By sequence homology 10 related proteins have been classified to this family. Src and Fyn are two members that have been perhaps more extensively studied in the nervous system. Subsequent studies of these proteins lead to the understanding that Src family kinases (SFK-s) are involved in various biological functions including cytoskeletal alterations, cell proliferation, adhesion, migration, but also survival and differentiation. The studies with knock-out mice show some specific functions for each family member, but also prove the redundancy of these kinases. SFK-s have been shown to be involved in signal

transduction from various types of receptors, including integrins, receptor protein tyrosine kinases, GPI-linked receptors and others (reviewed in Thomas and Brugge, 1997).

These above-mentioned biological functions of SFK-s involving the rearrangement of cytoskeleton and activity on receptor-dependent mechanisms suggest a role for these proteins also in neurite outgrowth. Indeed, growth cones contain Src and Fyn proteins and neurons cultured from mice deficient of either of these kinases show impairment in neurite outgrowth in substrate-dependent manner (Lee, 2005). Src also co-localizes with phosphorylated tyrosine markers at the tips of growth cone filopodia isolated from the neural tubes of developing mice. The activity of Src at these locations promotes neurite extension (Robles et al., 2005).

The exact mechanisms of Src´s role in this extension are not clear. Src is the main activator of FAK, a protein present in focal contacts and responsible for several activities involved in cellular movements and cytoskeletal dynamics in fibroblasts. The question of Src-FAK pathway in neurons is still poorly addressed (reviewed in Thomas and Brugge, 1997). There is some proof that Src gets locally activated upon tension in growth cones that appear after linkage of the growth cone membranes to the extracellular matrix. According to that model, activation of Src in turn leads to the strengthening of this linkage (Jay, 2001).

SFK-s have been shown to be involved in neurite growth in DRG neurons of adult mice upon stimulation with GDNF, NRTN and ARTN (Paveliev et al., 2004). GFL-s and their receptors have shown to trigger several signalling pathways depending on the form of co-receptors (soluble or membrane-bound) and localization of receptors in the membrane microdomains. Src signalling has been shown to be triggered upon GDNF binding to lipid-anchored GFRα1, upon which RET is recruited to the lipid rafts. Outside the lipid rafts, this complex is involved in other signalling pathways (Airaksinen and Saarma, 2002).

Cdk5 in axonal growth

Cyclin-dependent kinase 5 (Cdk5) is a small serine/threonine type of kinase. In spite of its name, it is one of the few Cdk-s that is not regulated by cyclins. Even though expressed also in kidney, testes and ovary, the activity of this kinase has been detected in postmitotic neurons in the developing and adult nervous system. The activity of Cdk5 requires association with one of the brain-specific regulatory subunits called p35 and p39. Cdk5 links extracellular signalling pathways and cytoskeletal systems to direct neuronal migration, axon growth and possibly neurosecretion (Smith et al., 2001).

Both Cdk5 and p35 have been show to localize in growth-cones and the activity of both is necessary for neurite outgrowth (Nikolic et al., 1996). A very elegant study in chick retinal ganglion cells revealed that Cdk5 is present in the whole cytoplasm of the dynamic growth cone while its activator p35 was detected only at stable parts. The activation of Cdk5 by p35 led to the stabilization of the growth cone and its transformation into a new distal axon, while in parts deficient of p35 the growth cone went through a collapse. This activity of growth cone stabilization and finally axon elongation was triggered by Cdk5 that phosphorylated the microtubule-associated protein 1. This phosphorylation lead to the stabilization of microtubules in the growth cone (Hahn et al., 2005).

Cdk5 is also activated in developing sympathetic neurons upon release of target-derived soluble GFRα1 isoform and GDNF stimulation. This phenomenon is necessary for axon terminals to be directed towards their targets to be innervated, as the axons of developing sympathetic neurons do not express membrane bound GFRα1 (Ledda et al., 2002).

Experimental part of the thesis

Aims of the study

A neuron on the verge of growing an axon has several decisions to make. It has to investigate the environment and find a permissive substratum. It has to follow the attracting neurotrophic factors secreted by targets and retract from repulsive sources. Taking into account all possible signalling molecules from the environment, the cell has to rearrange its cytoskeleton to first initiate and then elongate and turn the axon towards the right direction. All the attracting and inhibiting cues that a single neuron or a population of neurons receives trigger several signalling pathways inside the cell. The pathways interact and influence each other giving finally an implication to the final outcome: whether to initiate an axon, whether to elongate or retract, turn or proceed, etc.

The aim of this study is to segregate two inputs that lead to axonal growth and signalling pathways triggered by them. I try to find a role for laminin, as an extracellular matrix molecule, and neurotrophic factors, as diffusible molecules, in axonal growth, and find out to what extent the pathways downstream of these factors interact. The aim of this study is not only to reveal experimentally the signalling pathways from input to output, but also to create a logical model to describe it. The identified signalling molecules that participate in triggering axonal growth might be in the future used as targets for promoting axonal regeneration. I therefore try to set up *in vitro* experimental conditions to mimic the glial scar and in the future study the signalling pathways activated by inhibitory substratum.

Materials and methods

Isolation of primary DRG neurons

All animal experiments were done according to Finnish National Institute of Health guidelines and approved by the local Ethical Committee. Training at the lab animal course at the University of Helsinki was passed.

One-month-old NMRI or Balb/C mice from the animal house (Institute of Biotechnology, Helsinki University) were anaesthetized with CO_2 and sacrificed by cervical dislocation. Dorsal root ganglia were removed from the thoracic and lumbar segments of the spine, cleaned from nerves, incubated for 20 minutes at 37°C in 0.15% collagenase I, 0.025% trypsin and 0.4% bovine serum albumin, and triturated in cold Hanks Balanced Salt Saline (Gibco). Dissociated cells were plated on coverslips or 35-mm plates pre-coated with 1 mg/ml poly-ornithine overnight at 4°C and 100 ng/cm^2 (if not otherwise stated) laminin-1 (Invitrogen) for 4 hours at 37°C. Culture medium included in 1:1 relation F12 and DMEM (Dulbecco´s Modified Eagle Medium) media supplemented with Glutamax, penicillin, streptomycin (all from Gibco) and SATO serum supplement (0.35% bovine serum albumin, 60 ng/ml progesterone, 16 µg/ml putrescine, 400 ng/ml L-thyroxine, 38 ng/ml sodium selenite and 340 ng/ml triiodothyronine). NGF (Promega), GDNF, NRTN (PeproTech), Roscovitine and SU6656 (both from Calbiochem), integrin blocking antibodies (BD Biosciences) and TrkB-Fc (R & D) were added to cultures at the time of plating neurons if not otherwise stated.

Immunocytochemistry

Neurons were fixed at 12, 20, 40 or 90 hours after plating in 4% paraformaldehyde for 15 minutes at room temperature and permeabilized with 0.1% Triton X-100. Fixed coverslips were incubated with primary antibodies overnight at +4°C. For morphometric analysis, protein gene-product 9.5 rabbit antibody (Affiniti) was used which has been

shown to stain neurites and cell bodies of all sub-populations of DRG neurons (Wilson et al., 1988). Staining with secondary goat anti-rabbit antibodies Alexa-488 or Alexa-350 (Molecular Probes) was performed for 2 hours at room temperature in the dark. Coverslips were mounted in gelvatol before microscopic analysis of stained neurons.

Src dominant-negative recombinant adenovirus replication, infection and detection

A small aliquote of adenovirus stock expressing c-Src dominant negative form and green fluorescent protein (GFP) was a kind gift from David Kaplan (Popsueva et al., 2003). Amplification of viral stock was performed in human embryonic kidney cell-line HEK293 as follows. Cells were grown in T-75 flasks up to at least 70% confluency in DMEM medium supplied with fetal bovine serum, penicillin and streptomycin. Cells were infected with virus stocks for two hours in serum-free medium and grown until they were detached from the substratum. Cells were collected by centrifugation and broken via four freezing (on CO_2-ice and ethanol bath) and thawing steps. Virus particles were collected after centrifugation at 7700 rpm with SS34 rotor and concentrated in $CsCl_2$ gradient by centrifugation with SW41 rotor at 32000 rpm for 24 hours. The final virus stock was incubated in 5mM Tris-HCl (pH 8), 50mM NaCl, 0.05% bovine serum albumin and 25% glycerol. The final concentration of virus particles was tested in HEK293 cell-line and was measured as $4x10^8$ plaque forming units (PFU) per milliliter.

DRG neurons were infected at approximately 400 PFU/cell when plating. GFP signal could be detected at approximately 24 hours. Therefore neurite outgrowth assays were performed on low laminin concentrations, adding neurotrophic factors 40h after plating and fixing cultures at 90h time-point since plating. GFP-expressing adenoviral stock at the same dilution was used as a control (amplified by Antti Aalto). For neurite outgrowth assay, fixed samples were stained with antibodies PGP 9.5 and Alexa 350, infected and non-infected subpopulations were counted for neurite outgrowth assay.

In vitro kinase assay

Dissected neurons were plated on 35 mm plates that were beforehand pre-coated with poly-ornithine and with or without laminin, and lysed at 40h in lysis buffer (50mM Tris-HCl (pH 7.5), 150mM NaCl, 5mM EDTA, 1% Triton X-100, 1mM DTT, 1mM Na_3VO_4, Complete Mini protease inhibitors from Roche). Relative protein concentrations were measured by running the lysate on SDS-polyacrylamide gel and staining proteins with silver nitrate. Cdk5 from equal amounts of protein lysates was precipitated by Cdk5-specific antibody (Santa Cruz) and protein G Sepharose beads (Amersham). Kinase assay was performed for two hours at room temperature in kinase buffer (20mM Tris-HCl (pH 7.5), 1mM EDTA, 1mM $MgCl_2$, 1mM $MnCl_2$, 1mM DTT, 1mM Na_3VO_4, 20mM NaF, Complete Mini protease inhibitors from Roche) with precipitated Cdk5 beads, 10 μg of histone H1 (Upstate) and 5 μCi of [γ-^{32}P]ATP (GE Healthcare) per sample. 5x Laemmli buffer (300mM Tris-HCl (pH 6.8), 25% glycerol, 25% SDS, 0.015% bromophenol blue, 325mM DTT) was added to the whole reaction volume, which was then separated on 12% SDS-polyacrylamide gel. The histones were visualized with Coomassie staining (45% methanol, 10% acetic acid, 0.25% Coomassie blue), dried and autoradiographed on Fuji films. Quantification of radioactive signals was done using TINA quantification program (GmbH).

Glial scar modelling conditions

Poly-ornithine-coated coverslips (as described above) were dried at room temperature. 2 μl drops of aggrecan (0.3 mg/ml) mixed with laminin (5 μg/ml) in PBS were pipetted in the middle of the coverslip and dried again. The dried coverslips were coated with laminin (5 μg/ml) for 4 hours at 37°C. Neurons were plated as described above.

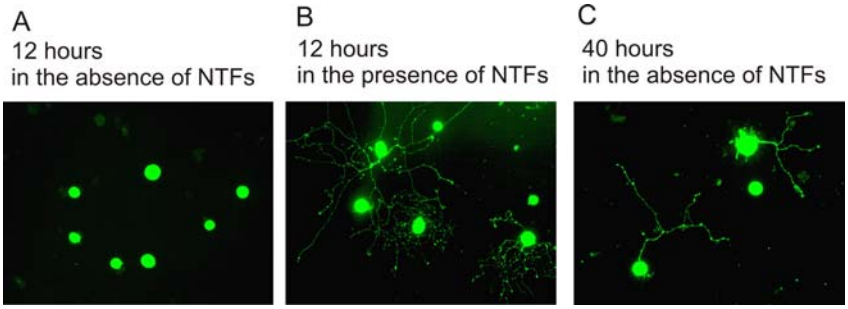
Image acquisition, morphometrical and statistical analysis

Gelvatol-embedded samples were viewed using the x20 magnifying objective of a Zeiss Axioplan 2 fluorescent microscope (Zeiss). Images were acquired with AxioCam CCD camera and AxioVision acquisition software (Zeiss). 100-500 neurons per sample were counted to calculate the percentage of process-bearing neurons. Cells with neurites longer than twice of the cell body diameter were considered as process-bearing. Statistical significance was calculated in all experiments for data from at least three independent experiments using one-way ANOVA (Excel, Microsoft). Error bars represent standard error of mean, symbols *, ** and *** represent $P<0.05$; 0.01 and 0.001, respectively.

Results

1. Identifying two types of neurite outgrowth in DRG neuronal cultures induced by laminin alone or laminin and NTF-s together

A growth-cone advances as a response to two guidance cues: ECM as a substrate and neurotrophic factors as soluble molecules. In the present work I used an experimental system which allowed me to discriminate between the NTF-dependent and –independent types of axonal growth in cultured DRG neurons from young adult mice.



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INPUT Ligand application		OUTPUT Axonal growth	
Laminin	NTF	12h in culture	40h in culture
-	-	-	-
+	-	-	+
-	+	-	-
+	+	+	+

Figure 6. *Dependence of neurite growth on laminin and/or neurotrophic factors at 12 and 40 hours after plating DRG neuronal cultures. (A) Representative neurons at 12 hours grown in the absence of NTF-s. (B) Representative neurons at 12 hours grown in the presence of NTF-s. (C) Representative neurons at 40 hours grown in the absence of NTF-s. In A-C neurons were cultured on precoated laminin substrate. (D) A summarizing table of requirements for ligand application as input in order to promote axonal growth as output. "+" refers to the availability of ligand and positive axonal growth as a result. "-" refers to the absence of ligand and lack of axonal growth as a result to the used conditions. NTF – neurotrophic factor.*

Neurons were cultured on coverslips precoated with laminin. I found that on the described conditions cultured DRG neurons grew neurites by 12 hours after plating when incubated with NGF, NRTN or GDNF (Figure 6B). No neurites were observed by that time without any neurotrophic factor (Figure 6A). Laminin alone was capable of promoting outgrowth by 40 hours without addition of neurotrophic factors (Figure 6C). Importantly, laminin was necessary for outgrowth at both time-points, as neurotrophic factors alone did not trigger any neurite growth by these time-points. A summarizing table for outgrowth as response to different conditions by different time-points is presented in figure 6D.

2. Chemical inhibitors of Src family kinases and Cdk5 affect NTF-dependent and -independent neurite outgrowth differently

Previously I distinguished two types of neurite outgrowth: one dependent on laminin and neurotrophic factors and the other one dependent on the ECM molecule laminin alone. I believed that these outgrowth types were triggered via two different signalling pathways. To investigate this further, I tested inhibitory chemicals that have been previously shown to specifically inhibit signalling molecules of interest (Bain et al., 2003).

I found that SU6656, an inhibitor for Src family kinases, inhibits outgrowth promoted by either NGF, NRTN or GDNF on laminin substrate in a concentration-dependent manner (Figure 7A). This same inhibitor did not have an effect on laminin-dependent outgrowth observed at 40 hours even at higher concentrations (Figure 7B).

I also tested Roscovitine, an inhibitor of Cdk5, and found it specifically blocking laminin-dependent outgrowth at 40 hours (Figure 7D). Roscovitine did not affect outgrowth induced by three neurotrophic factors at 12 hours (Figure 7C).

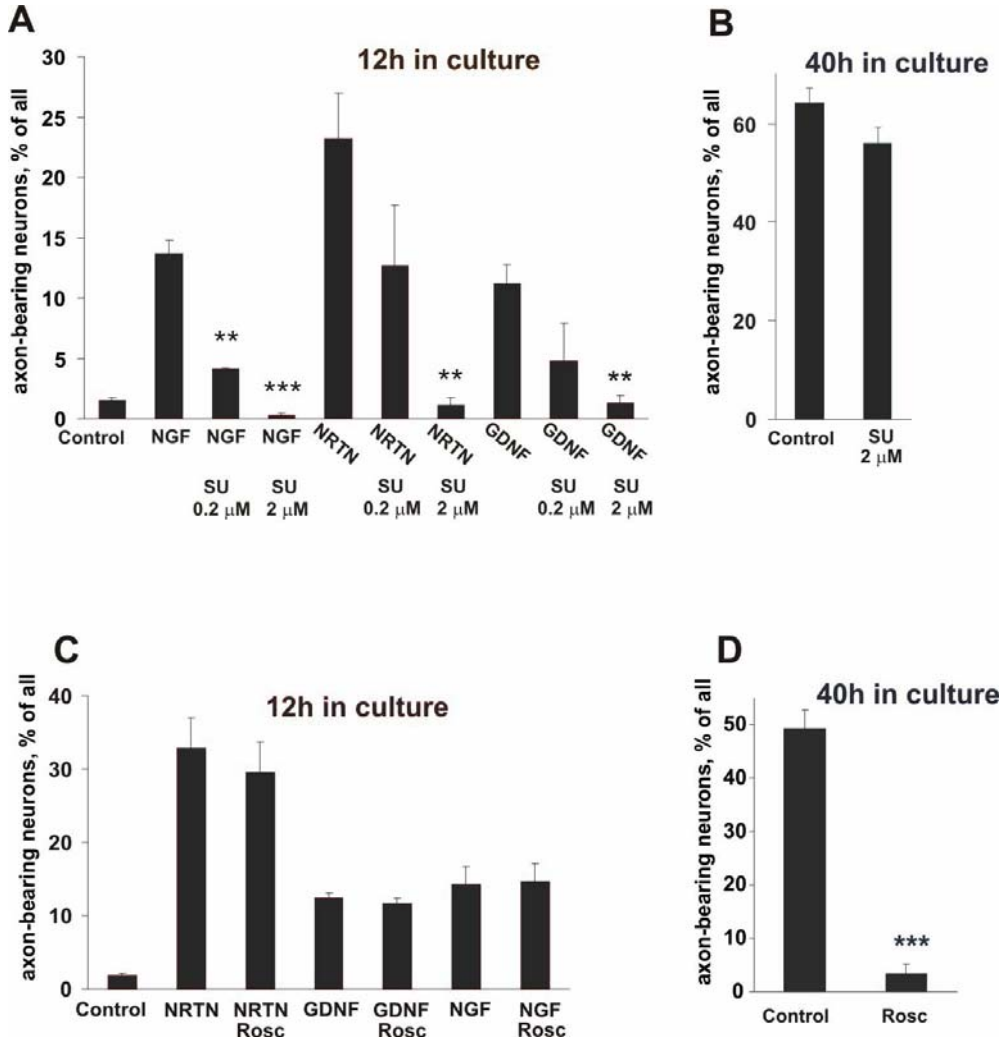

Figure 7. *Neurite outgrowth inhibition by chemical inhibitors SU6656 and Roscovitine. (A) An SFK inhibitor SU6656 reduces significantly neurite outgrowth by 12 hours in a concentration-dependent manner. This outgrowth is triggered by NGF, NRTN or GDNF on laminin substrate. (B) The same inhibitor SU6656 has no significant effect on NTF-independent outgrowth observed at 40 hours. (C) The Cdk5 inhibitor Roscovitine has no effect on outgrowth at 12 hours triggered by three different neuotrophic factors. (D) Roscovitine significantly inhibits outgrowth triggered by laminin by 40 hours. Controls in A and C mean neurons cultured on laminin for 12 hours without any neurotrophic factors or inhibitors. Controls in B and D mean neurons cultured on laminin pre-coated coverslips for 40 hours without incubation with any inhibitor. GDNF – glial cell-line derived neurotrophic factor, NRTN – neurturin, Rosc – Roscovitine, SU – SU6656*

3. Transduction of neuronal cultures with adenovirus expressing Src dominant negative protein inhibits NTF-dependent outgrowth

To address the problem of specificity of SFK inhibition by SU6656, I used an adenoviral vector for expressing the dominant negative form of Src (SrcDN) and GFP under different cytomegalovirus (CMV) promoters. I transduced cells with adenoviral particles expressing the mentioned proteins, added NGF and NRTN and measured neurite outgrowth of the transduced and non-transduced neurons. Adenovirus-positive cells were distinguished by GFP expression and neurites were counted after staining the cultures with PGP 9.5 and Alexa-350 (visualized in Figures 8C and 8D). A similar adenoviral vector expressing only GFP was used as a control.

As the expression of GFP was not detectable in neurons before 24-40 hours after transduction, I could not use the 12-hour time-point to observe neurotrophic factor-dependent outgrowth as previously. I had to reduce the amount of laminin 5-10 times from the usual to inhibit the speed of laminin-induced neurite outgrowth before adding neurotrophic factors. Cultures were infected with adenovirus at the time of plating, but NTF-s were added 40 hours later (cultures at 40 hours are visualized in Figures 8A and 8B). In this way I could be sure that Src kinase was inhibited before signalling events from NTF-s were triggered. Cultures were fixed at 90 hours after plating and neurite outgrowth was counted. In this way I could postpone the effect of laminin on neurite growth and observe the effect of SrcDN protein on neurotrophic factor-dependent outgrowth. In the control samples for NTF-independent outgrowth higher concentrations of laminin were used.

As a result of this experimental set-up I could distinguish between two types of outgrowth with slightly altered conditions than previously. I observed that SrcDN inhibited NTF-dependent outgrowth (Figure 8E), while laminin-dependent outgrowth was not reduced in comparison to cells transduced with GFP-expressing adenovirus (Figure 8G). As a second control, I used non-infected cells in the samples that were

transduced with SrcDN-expressing adenovirus and observed no significant inhibition of neurite outgrowth in this neuronal population (Figure 8F).

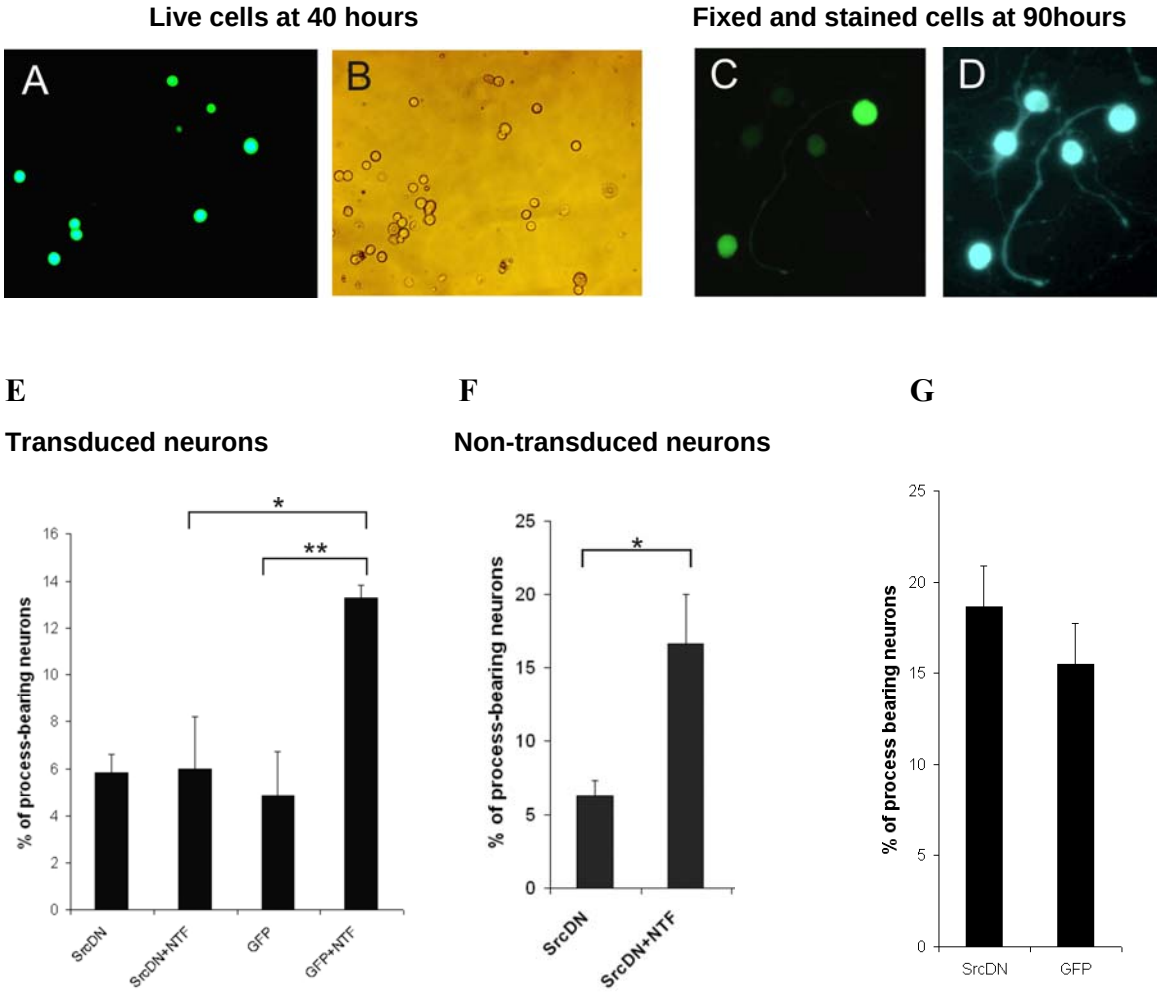

Figure 8. *Expressing SrcDN and/or GFP via adenoviral vectors in DRG neurons. (A) A representative image of live neurons at 40 hours in culture transduced with SrcDN- and GFP-expressing adenovirus. Visualization of transduction efficiency by GFP signal. (B) All neurons in the same field as in A in phase contrast field. (C) A representative view of DRG neurons transduced with GFP-expressing adenovirus and fixed at 90 hours. Visualization of transduction efficiency through GFP channel. (D) All neurons in the same field as in C stained with PGP 9.5 and Alexa-350 antibodies. (E) Transducing neurons with SrcDN-expressing adenovirus inhibits NTF-dependent outgrowth by 90 hours. Expression of GFP in the same system does not have an effect on neurite outgrowth. (F) Non-infected cells in the same samples as in E did not show inhibition of neurite outgrowth as response to SrcDN expression. (G) The percentage of process-bearing neurons is comparable in the samples infected with SrcDN- and GFP-expressing adenovirus without NTF-s. GFP – green fluorescent protein, NTF – neurotrophic factors, SrcDN – dominant negative form of Src.*

4. Cdk5 activity is impaired in DRG neurons deprived of contact with laminin matrix

I previously showed that Roscovitine, the inhibitor of Cdk5, decreases specifically laminin-dependent, but NTF-independent outgrowth. Subsequently I also wanted to know if signalling from laminin somehow influences the activity of Cdk5. To address this question, I performed an *in vitro* kinase assay. I precipitated Cdk5 from DRG lysate prepared from neurons that were grown for 40 hours on laminin substrate or without laminin. For this I used a Cdk5-specific antibody for which specificity had been proved in previous works (Ledda et al., 2002; Pareek et al., 2006). The activity of the precipitated Cdk5 was assessed upon its ability to transfer the gamma-phosphate moiety of radioactive ATP molecules to histone H1 that I used as a substrate for the reaction.

As a result of this experiment, I found that the activity of Cdk5 was remarkably enhanced in neurons grown on laminin compared to the ones grown on poly-ornithine pre-coating only (Figure 9B). Before precipitation of Cdk5, the initial concentration of total protein was normalized. As shown in Figure 9A, the amounts of total histone H1 in both samples were also equal. I quantified the radioactive signal from the autoradiography from three independent experiments. The results are graphically presented in Figure 9C as a relative percentage of kinatic activity of Cdk5 induced by laminin. I measured that the average inhibition of the kinatic activity of Cdk5 without laminin substrate was approximately 33%. Therefore I conclude that signalling from laminin enhances Cdk5 activity possibly leading to the promotion of axonal growth.

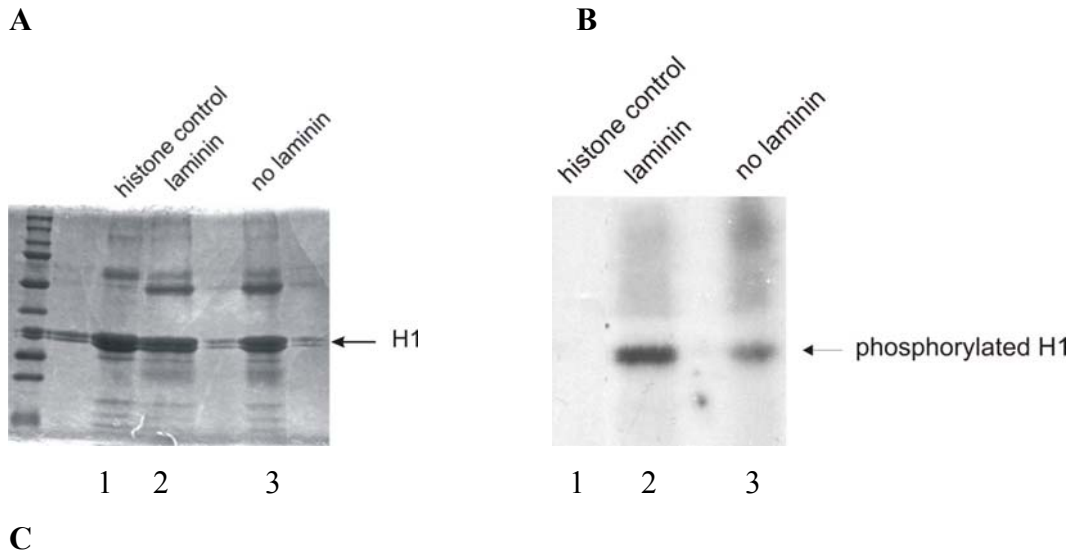

Figure 9. *Assessing Cdk5 activity by in vitro kinase assay. The experiment was performed as described in the text and in "Materials and methods". (A) The total amounts of histone H1 were equal in both reactions when comparing lanes 2 and 3. (B) Cdk5 isolated from cells grown on laminin phosphorylated histone H1 more efficiently (lane 2) than the Cdk5 isolated from cells previously grown on substrate without laminin pre-coating (lane 3). Histone control in both figures refers to pure histone H1 loaded on the gel as a marker for size. (C) Quantified results from three independent experiments presented as the percentage of laminin-induced kinatic acivity of Cdk5. H1 – histone H1*

5. Neurite outgrowth in our established system is dependent on integrin β1 signalling

It was shown already more than a decade ago that integrins are responsible for mediating signals from the ECM molecule laminin to axon terminals and triggering neurite outgrowth in DRG neurons. Integrin β1 has been shown to be the central molecule for this activity, forming receptor complexes with different α subunits (Tomaselli et al., 1993).

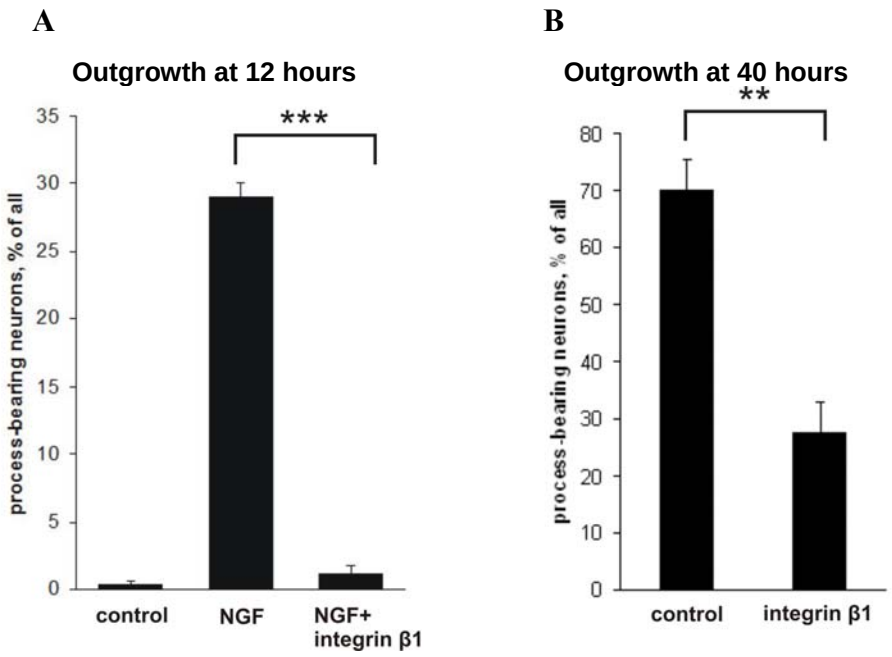

Figure 10. *Blocking integrin β1 by function-blocking antibodies reduced outgrowth at 12 hours in the presence of NGF (A) and at 40 hours without NGF (B). Control in both figures refers to samples treated with equal amounts of PBS and fixed at the referred time-points. NGF – nerve growth factor.*

I described earlier that in our model DRG neurons generate neurite outgrowth by 12 or 40 hours depending on the conditions. Both of these outgrowth types were dependent on laminin matrix. Therefore I suggested that blocking β1 integrin should inhibit neurite outgrowth in this system. I added β1 integrin blocking antibodies to cell culture medium at the time of plating and observed that by both, 12 hours (Figure 10A) and 40 hours (Figure 10B) remarkably fewer neurons were bearing neurites.

6. Laminin-induced NTF-independent neurite outgrowth is not resulting from autocrine or paracrine actions of BDNF

I have identified two types of neurite outgrowth in DRG neuronal system. I can distinguish between these two types temporally. I have shown that by 40 hours I get neurite outgrowth that is believably induced by laminin signalling through Cdk5 (see chapter 4 and Figure 9). However, it has been described that adult DRG neurons express

both BDNF and its receptor TrkB and form autocrine and/or paracrine loops to affect cellular activities like survival (Acheson et al., 1995). To make sure that the neurite outgrowth observed at 40 hours is not a result of such loop, I blocked BDNF signalling by using TrkB soluble receptor body (TrkB-Fc). This protein has been shown previously in our lab to be specific for inhibiting BDNF signalling by competing with intact membrane-bound TrkB receptor and scavenging BDNF (Rivera et al., 2002).

I applied TrkB-Fc into the culture medium at the time of plating. By 40 hours in culture I did not observe any remarkable difference in the amount of process-bearing neurons compared to the control cultures where TrkB-Fc was not added (Figure 11).

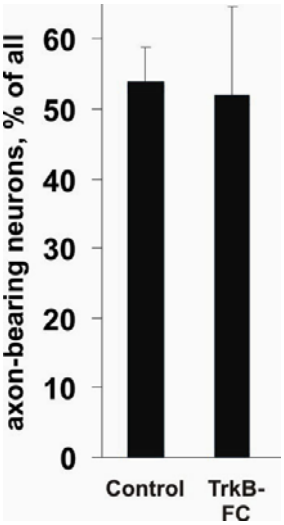

Figure 11. *Inhibiting BDNF-TrkB signalling does not alter the proportion of process-bearing neurons by 40 hours in culture. TrkB-Fc protein was applied at the time of plating the neurons. Control samples were untreated with TrkB-Fc.*

7. Creating a Boolean model for SFK- and Cdk5-dependent signalling pathways and their integration

Until this point of the current study I have shown the linear dependence of ligand-signalling molecule-outgrowth type of pathway. As laminin is a crucial input for both of the systems, it is obvious that there is a cross-talk between these pathways triggering neurite outgrowth in NTF-dependent or -independent manner. To better characterize the relationships between extracellular inputs, intracellular signalling pathways and axonal growth as the common output under study, I propose a Boolean model. This simple

scheme consists of two inputs: laminin and neurotrophic factors; and one output that is axonal growth. Links that characterize the interactions between input and output are functions "AND" and "OR". "AND" in this model refers to the necessity of all signalling pathways leading towards this function and giving a certain output. Function "OR" means that any of the inputs is sufficient to give the resulting output (Huang and Ingber, 2000).

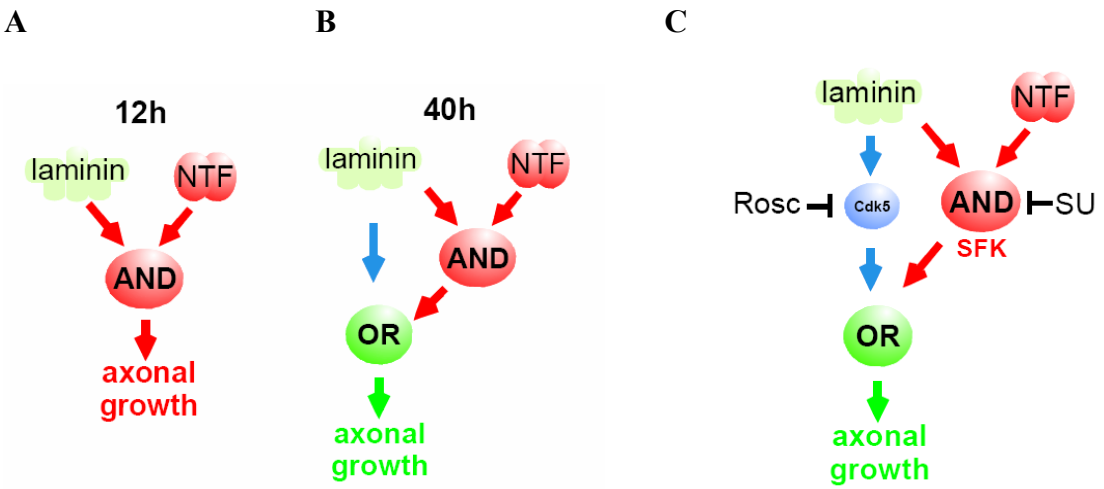

Figure 12. *Boolean network describing the interactions of two pathways leading to axonal growth in DRG neurons. (A) At 12 hours both laminin and NTF-s are necessary for triggering neurite outgrowth. (B) At 40 hours laminin alone (depicted by blue arrows) or together with NTF-s (depicted by red arrows) can independently trigger axonal growth. (C) Placing the identified signalling molecules SFK and Cdk5 that are activated downstream of extracellular inputs into the network. More details are described in the text. NTF – neurotrophic factors, Rosc – Roscovitine, SFK – Src family kinases, SU – chemical SU6656*

When using the Boolean logics on our system of two types of outgrowth, it would look as depicted in Figure 12 (A and B). At 12 hours in culture, neuronal cell needs to receive a signal from both laminin and NTF-s, and that is the reason why "AND" factor is used at their crossing point (red arrows in all schemes in Figure 12). At 40 hours either the previously described system (depicted in red arrows in Figure 12B) or laminin alone (blue arrows on the same scheme) are independently sufficient to lead to axonal growth. Therefore the factor "OR" is used before the output. In Figure 12C the interactions of

inputs leading to axonal growth are presented together with activated signalling molecules downstream of these inputs. As Roscovitine inhibits neurite outgrowth dependent on laminin only, I propose that Cdk5 is placed in this system between laminin and the factor "OR" on the blue pathway. SFK-s are probably the crossing point where signalling from laminin and neurotrophic factors interact (the "AND" factor on the red pathway), because SU6656 and SrcDN inhibit specifically NTF- and laminin-dependent outgrowth. That kind of modelling allows to create a better overview of interacting signalling pathways and to choose specific targets for therapeutic interventions.

8. Setting up conditions that mimic the glial scar *in vitro*

Patients who suffer from paralysis have often injured their spinal cord or sensory nerves. A lot of effort has therefore been put into studies of DRG neuronal regeneration. It has been shown that after injury these neurons can regenerate and innervate their peripheral targets, but regeneration into the central nervous system is impaired. One of the reasons for this is the hostile environment created by the reactive astrocytes at the glial scar region. Astrocytes secrete CSPG-s that inhibit axonal growth (Bradbury et al., 2000).

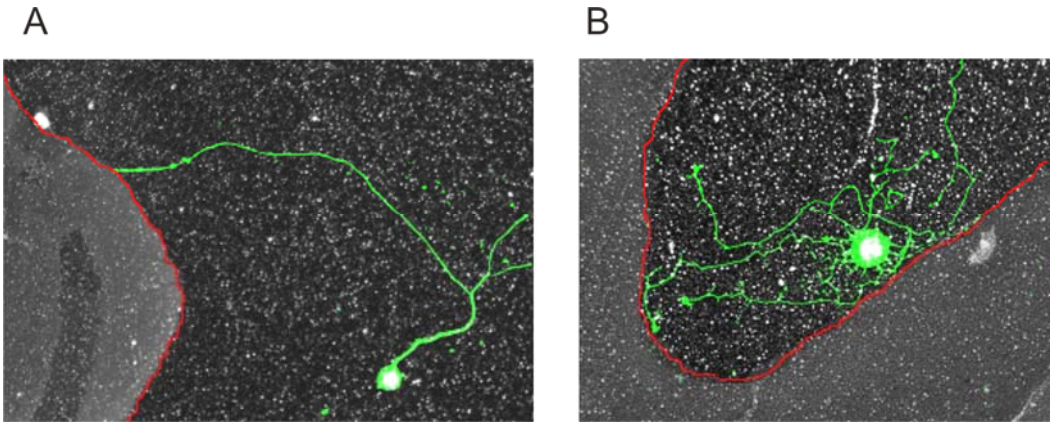

Figure 13. *An in vitro model for the glial scar. The coverslips were pre-coated with aggrecan-laminin spots, dried and coated with laminin at high concentrations. (A) A representative neuron with its axon arrested at the laminin-aggrecan border. (B) A representative neuron that has turned its axons away from the laminin-aggrecan border. Green – DRG neuron, red – laminin-aggrecan border.*

I have created an *in vitro* model for studying the glial scar, where substrate of high laminin concentration gives a positive cue for axonal growth and aggrecan, an CSPG molecule, provides a negative cue. I observed two phenomena in this system: growth cone arrest (Figure 13A) and axon repulsion (Figure 13B) at the laminin-aggrecan border. In this system I would be further interested in the effect of NTF-s on the repulsive property of aggrecan and the intracellular signalling pathways activated by these inhibitory cues.

Discussion

Identifying signalling pathways, the extent of their cross-talk and overlap is important in many aspects. Firstly, it would give a better understanding and insight into how difficult processes are managed by a single cell. This would be the biological value of the information. Secondly, it would help to find more specific targets for therapeutic interventions that would lead to establishing more efficient cures for several diseases. Modern therapeutic targeting involves usually three broader aspects of cell signalling: the receptors for extracellular cues, intracellular signalling pathways and intercellular communication (Rajasethupathy et al., 2005).

In this work a simpler model was set up for only two extracellular cues, laminin and neurotrophic factors, that would lead to the same output – axonal growth. It was observed that laminin alone and laminin together with neurotrophic factors elicit neurite outgrowth via different pathways by different time-points (Figure 6). This temporal dissimilarity gave a possibility to differentially distinguish the pathways downstream of the two inputs that would be activated.

NTF-s have been well known for supporting cell survival and promoting axonal growth. NGF and GDNF have been successfully investigated for their ability to promote functional regeneration of DRG axons into the spinal cord after dorsal root avulsion (Ramer et al., 2000). In the model described in this study NTF-s NGF, GDNF and NRTN also promote axonal growth. Compared to laminin alone, the positive extracellular matrix cue together with NTF-s triggers axonal growth faster. This temporal difference is achieved by the activation of Src family kinases by laminin and NTF interaction (Figures 7A and 7B). The identification of SFK-s at this crossing point of two inputs might reveal a mechanism for nerve regeneration as both the levels and activity of Src have been shown to increase after peripheral nerve injury (Ignelzi et al., 1992). It seems that in our system the activity of SFK-s is important for axonal growth as SU6656 is an inhibitor of the kinatic activity of SFK-s, competing with ATP binding (Blake et al., 2000), and dominant negative form of Src impairs the activity of naturally occurring endogenous

Src. Both of these methods were specific for reducing NTF-dependent outgrowth, at the same time not affecting NTF-independent outgrowth (Figures 7 and 8).

The Cdk5 inhibitor Roscovitine, characterized in DeAzevedo *et al* in 1997 (DeAzevedo et al., 1997), significantly reduced the outgrowth in response to laminin in our model (Figure 7D). Cdk5 has been shown to regulate neuronal migration and axonal growth in the neurons of the central and peripheral nervous system (Dhavan and Tsai, 2001). In this study it was shown that laminin alone is responsible for activating Cdk5 (Figure 9B) and this pathway is not involved in NTF-dependent axonal growth (Figure 7C). Whether the difference in Cdk5 activity is a result of upregulation of Cdk5 expression or that of its activator p35, or if Cdk5 is present in an inactive state in the cells grown without laminin, is a material for further study and this question was not addressed in this thesis.

The necessity of laminin input for both pathways refers to the fact that a neuron needs permissive substrate to initiate axon growth. The signalling from laminin is transferred via integrin β1, which forms dimers with different α subunits (Tomaselli et al., 1993). Blocking antibodies directed against integrin β1 significantly inhibited laminin-induced neurite outgrowth at both time-points in our model (Figure 10A and 10B). At 12 hours the inhibition of outgrowth was nearly complete. At 40 hours, however, about a third of the neurons compared to the control sample were still able to initiate and elongate axons after blocking integrin β1. This would suggest that there are some additional receptor types that also contribute to axonal growth triggered by laminin matrix. The other possibility is that the inhibition of integrin β1 was not complete due to the instability of the blocking antibody over time.

DRG neurons have been shown to endogenously express BDNF and its receptor TrkB (Acheson et al., 1995). In this way they could provide paracrine or autocrine loops for promoting cellular activities including axonal growth. I blocked BDNF signalling by adding chimeric soluble TrkB-Fc and found that BDNF autocrine or paracrine signalling had no effect on neurite outgrowth (Figure 11). The question of endogenous synthesis and secretion of neurotrophic factors and their effect on neurite growth in DRG neurons

was addressed also by other colleagues. Blocking NGF, GDNF and glial cell signalling did not affect laminin-induced and NTF-independent outgrowth. Applying conditioned medium from 40 hours old culture to a newly plated one also did not make any difference in the efficiency of neurite growth (Paveliev et al., unpublished manuscript). Therefore we believe that the effects of Cdk5 activation and neurite outgrowth by later time-points are induced by laminin alone.

Laminin is considered to be a positive cue for axonal growth in DRG neurons. However, in an organism it is not the only extracellular matrix molecule available. The combinations of different guidance cues can give a summarizing effect to a cellular activity and negative cues can mask the effect of positive ones. Exactly that is the case in the glial scar after nerve lesion in the CNS. Besides the availability of laminin in glial scar tissue, reactive astrocytes secrete inhibiting CSPG-s and this is one of the reasons for deficiency of axonal regeneration into the CNS (Mckeon et al., 1991; Acheson et al., 1995). A similar event is depicted in Figure 13 of this thesis, where growth cone of an adult DRG neuron arrests (Figure 13A) or turns (Figure 13B) from the border of laminin-aggrecan area into the only laminin-containing area. It is obvious that laminin induces outgrowth that involves Cdk5 activity, but this is not enough for overcoming inhibitory signals, even when laminin is also present at the repulsive areas.

I suggested a Boolean model that would describe the situation in the described system, where two types of input lead to the same output via two different pathways that may interact (Figure 12). That kind of modelling is helpful in determining the crossing points of the pathways and identifying the upstream-downstream relationships of signalling molecules. It also gives an insight into how complicated the signalling networks can be when the cell faces different conditions, which is a normal situation in a complex multicellular organism. Only one example is the fact that pathways activated by Cdk5 by laminin are not enough to promote axonal growth into aggrecan-coated inhibitory environment. Possibly other pathways should be activated there to make the axonal penetration and full regeneration possible.

Conclusions

In the experimental part of the thesis I showed that:

1. Two independent pathways trigger axonal growth in dorsal root ganglion neurons. Both pathways are dependent on laminin. Co-stimulation with neurotrophic factors and laminin triggers axonal growth at 12h in culture (NTF-dependent growth). In the absence of NTFs laminin activates axonal growth observed at 40h in culture (NTF-independent growth).
2. The cross-talk between neurotrophic factors and laminin affects Src family kinases. It was shown that inhibiting Src activity by dominant negative protein expression or by chemical inhibitor SU6656 reduced NTF-dependent neurite growth. The NTF-independent axonal growth does not require signalling through Src family kinases.
3. The NTF-independent axonal growth requires activation of cyclin-dependent kinase 5. The activity of Cdk5 was measured in an *in vitro* kinase assay, the effect on outgrowth was tested by using Roscovitine, a specific inhibitor of Cdk5. Inhibition of Cdk5 activity did not have any effect on NTF-dependent outgrowth.
4. Both outgrowth types were dependent on laminin receptor integrin-β1 signalling.
5. NTF-independent outgrowth did not derive from the autocrine or paracrine actions of endogenous BDNF.
6. As a result of the performed experiments, a Boolean model was suggested to describe the interaction of signalling pathways triggered by the two types of inputs leading to axonal growth.
7. An *in vitro* glial scar model was described, where growth-cone arrest and axonal turning were demonstrated at the border of permissive and non-permissive substrata.

This work has hopefully improved the understanding of signalling cross-talk between different input types and explained the necessity of creating logical models according to obtained experimental results. I believe that this study would help to specifically address complicated targeting problems.

Summary

The sensory nervous system is a mean for an organism to receive information from its surrounding environment and internal organs. Dorsal root ganglion (DRG) neurons are responsible for the sensation of pain, temperature, mechanical stimuli, and the perception of body position. Paralysis that results from injuries of the central projections of DRG neurons is so far effectively incurable and leads to life-long disability. The problem of incurability derives from the fact that DRG neurons are unable to regenerate their axons back into the spinal cord due to the hostile environment in the central nervous system. Therefore it is very important to study the mechanisms of axonal growth of these neurons and describe the signalling pathways triggered by positive and negative guidance cues that a growing axon terminal faces.

The current thesis addresses some of the questions of molecular mechanisms that underlie axonal growth in sensory neurons. The literature review gives a summary of the general knowledge of the role of DRG neurons in the sensory nervous system. The importance of neurotrophic factors and extracellular matrix molecules on axon growth and guidance are described with the emphasis on the molecular mechanisms known to promote axonal growth.

In the experimental part an *in vitro* model for neurite growth in DRG neurons was set up and two types of signalling pathways were identified that both lead to axonal growth. One of the pathways is triggered by laminin, which activates Cdk5 and the other one is triggered by neurotrophic factors together with laminin with the involvement of Src family kinases. A logical model based on Boolean algebra was proposed that describes the exact upstream-downstream positions of the identified molecular players and their interactions. Finally, an *in vitro* model for the glial scar was set up that would mimic the extracellular environment after the CNS injury. This model is meant for the study of determining the molecular targets for promoting axonal growth into the hostile environment of the injured central nervous system.

Kokkuvõte

Sensoorse närvisüsteemi ülesandeks on võtta vastu ja edastada kesknärvisüsteemile organismi välis- ja sisekeskkonnast saabuvaid signaale. Seljajuure ganglionite (DRG) neuronid vastutavad selliste aistingute edastamise eest nagu valu, mehhaaniline ärritus, temperatuur, kehaosade orientatsioon jms. DRG neuronite seljaajju suubuvate aksonite kahjustus viib täna veel enamasti ravimatu halvatuseni, kuna need aksonid ei ole võimelised seljaajju tagasi kasvama kesknärvisüsteemis leiduvate regeneratsiooni pidurdavate molekulide tõttu. Sel põhjusel on väga oluline uurida aksonite kasvu tagavaid molekulaarseid mehhanisme ning erinevaid aktiveerivaid ja tõkestavaid signaalmolekule, mida aksoni kasvukoonus oma teel kohtab.

Käesolevas magistritöös adresseerisin mõningaid küsimusi, mis puudutavad sensoorse närvisüsteemi regeneratsiooni mehhanisme. Kirjanduse ülevaade keskendub seljajuure ganglionite neuronite iseloomustamisele ja nende rolli kirjeldamisele sensoorses närvisüsteemis. Suuremat rõhku on pandud juba teadaolevale informatsioonile neurotroofilistest faktoritest, rakuvälisest maatriksist ja rakusisestest signaaliradadest.

Töö praktilises osas kirjeldasin sellise mudeli arendamist, mille põhjal uuriti *in vitro* DRG neuronite aksonite kasvu. Leidsin kaks erinevat signaalirada, mis vahendavad aksonite kasvu. Ühte neist aktiveerivad neurotroofilised faktorid koostoimes rakuvälise maatriksi valgu laminiiniga ning selles rajas osalevad Src perekonna kinaasid, mille ensümaatiline aktiivsus on aksoni kasvuks oluline. Teine signaalirada aktiveeritakse ainult laminiini poolt ilma neurotroofiliste faktorite osaluseta. Erinevalt esimesest signaalirajast on siin oluline Cdk5 kinaasi osalus, aga mitte Src kinaasid. Püstitasin ka signaaliradade interaktsioone ja nendes osalevate elementide üksteise suhtes paiknemise formaliseeritud mudeli kasutades Boole´i algebrat. Lõpuks kirjeldasin ka *in vitro* gliia armkudet matkivat eksperimentaalset süsteemi. Selline aksonite kasvu pidurdav kude tekib kesknärvisüsteemi vigastuse tagajärjel. Mudel on seega mõeldud uurimaks molekulaarseid mehhanisme, mis aitaksid vigastatud aksoni kasvukoonusel ületada regeneratsiooni inhibeerivaid molekule ja taastuda sihtkohta tagasi.

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Appendix

"Neurotrophic factors switch between two signalling pathways that trigger axonal growth" article manuscript by Paveliev M, Lume M, Velthut M, Phillips M, Arumäe U, Saarma M. Submitted to Journal of Cell Science.

Title: Neurotrophic factors switch between two different signaling pathways that trigger axon growth in sensory neurons

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The condensed title: NGF/SFK signaling for axonal growth

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Abstract

Integration of multiple inputs from the extracellular environment such as extracellular matrix molecules and growth factors, is a crucial process for cell function and information processing in multicellular organisms. Here we demonstrate that co-stimulation of dorsal root ganglion neurons with neurotrophic factors (NTFs) (GDNF, neurturin or NGF) and laminin leads to axonal growth that requires activation of Src family kinases (SFKs). A different, SFKs-independent signaling pathway evokes axonal growth on laminin in the absence of the NTFs. We propose and experimentally verify a Boolean model of the signaling network triggered by NTFs and laminin. Our results demonstrate that NTFs provide an environmental cue that triggers a switch between separate pathways in the cell signaling network.

Introduction

There is a major unsolved question of the modern cell biology: how does a cell convey environmental information (receptor inputs) into adaptive behavioral programs (motility, gene expression, etc.) (Bray, 1995). A single cell has hundreds of different types of receptor proteins on its surface, making complex combinatorial relationships
possible between signaling inputs and signaling network behavior. It remains largely unclear how different combinations of receptor signaling inputs are differentially recognized by the signaling network in such a way as to trigger execution of particular cellular events like gene transcription or cytoskeletal rearrangement. Signaling pathways downstream of different receptors are known to crosstalk at many levels and to converge at a limited number of signaling targets. A complex network of signaling pathways mediates regulatory effects of neurotrophic factors (NTFs) and extracellular matrix (ECM) molecules on a wide spectrum of processes in neurons including synaptic transmission, axonal growth, and posttraumatic regeneration. Many signaling elements of those pathways have been characterized and activatory/inhibitory effects of particular elements on cell function have been described. At the same time the integral functioning of the signaling network is just beginning to be addressed. It remains largely unclear how distinct pathways are orchestrated in the integrated network to produce highly specific cell responses to receptor stimulation.

A critical obstacle to progress in our understanding of cell signaling networks is the lack of a standard unambiguous language allowing quantitative description and analysis of the network behavior (Lazebnik, 2002). The formalism of Boolean algebra was previously used to analyze a genetic network in the specific case of the bacteriophage lambda lysis-lysogeny decision (McAdams and Shapiro, 1995), and it was theoretically predicted that protein kinase signaling networks can be described in Boolean terms (Bray, 1995; Huang and Ingber, 2000; Bhalla, 2003). Moreover, signaling molecules with particular gating behaviors have been generated by means of protein domain recombination (Dueber et al., 2003; Dueber et al., 2004). In the present work we considered a Boolean networks formalism as a framework for experimental design and the interpretation of experimental data on a signaling network behavior.

ECM molecules and NTFs are two classes of extracellular ligands that have crucial effects of axonal growth both *in vivo* and *in vitro* (Cai et al., 1999; Ramer et al., 2000; Chen and Strickland, 2003). Treatment with NTFs (especially nerve growth factor (NGF) and glial cell line - derived neurotrophic factor (GDNF)) is the only known way to induce axonal regeneration into the root entry zone of the spinal cord after dorsal root avulsion (Ramer et al., 2000; Schwab, 2000). GDNF also promotes axonal regeneration in propriospinal neurons after spinal cord injury (Iannotti et al., 2003). Neurturin (NRTN), which is a close homolog of GDNF, restores hindlimb cutaneous innervation in a mouse model of diabetes (Christianson et al., 2003) and activates axonal growth in Dorsal Root Ganglion (DRG) neurons *in vitro* (Paveliev et al., 2004). NGF activates axonal growth by signaling through TrkA receptor tyrosine kinase (Kaplan and Miller, 2000). GDNF and NRTN bind specifically to GPI-linked receptors - GDNF Family Receptor α1 (GFRα1) and GFRα2, respectively, and the ligand-receptor complex activates transmembrane receptor tyrosine kinase, Ret (Airaksinen and Saarma, 2002). Laminin is an ECM molecule that regulates axonal growth by activating integrins (Tomaselli et al., 1993) and has a crucial impact on axonal regeneration in sciatic nerve (Chen and Strickland, 2003). Peripheral axons were shown to regenerate on laminin matrix in acellular grafts of sciatic nerve (Krekoski et al., 2001). Several intracellular signaling pathways are known to mediate positive effect of NTFs and laminin on axonal growth (Paglini et al., 1998; Liu and Snider, 2001; Liu et al., 2002; Markus et al., 2002; Laforest et al., 2005), but the integral behavior of the signaling network converting receptor activation into axonal growth remains largely unknown (Tucker et al., 2005). Importantly, co-signaling by soluble NTFs and substrate-bound ECM molecules integrates two significantly different types of intercellular communication – long-range messages from remotely diffusing messangers, and those which are highly localized to matrix.

Here we demonstrate that the ECM molecule laminin is differentially recognized by neurons in the presence of, versus the absence of NTFs. The essence of this difference is laminin-dependent activation of two different signaling pathways, each leading to axonal growth. NTFs activate the pathway involving Src-family kinases (SFKs), while in the absence of NTFs axonal growth is independent of the SFKs activity. We then propose

and experimentally verify a Boolean model describing differential contribution of the NTF- and laminin-triggered pathways to the converging signaling network.

Results

In our experiments NGF, GDNF or NRTN (100ng/ml) trigger axonal growth in DRG neurons on laminin-precoated substrate at 12h in culture (Fig. 1 Ab). At 40h in culture, neurons produce axons without NTFs treatment (Fig. 1 Ac), but laminin precoating is absolutely required. If the substrate (glass coverslips) is precoated with poly-DL-ornithine without laminin, neurons fail to produce axons both in the presence and in the absence of NTFs (assessed at 12h and 40h time points, Fig. S1). Borrowing the Truth table formalism from Boolean Logic we can fully describe this input/output (ligand/ outgrowth) dependence (Fig. 1 B). At 12h in culture the two inputs (laminin and NTF) are connected to the output through the conjunctive (AND) operation – the output is ”True” only if both inputs are present (Fig. 1 C). At 40h in culture the output is switched on by a single input (laminin) – the blue branch in Fig. 1 D. Fig. 1 D summarizes two types of outgrowth - NTF-dependent (red branch) and NTF-independent (blue branch). According to the table (Fig. 1 B) the two branches converge on the disjunctive operation (OR) to produce, again, the output axonal growth. Here “OR” means that each branch alone is sufficient to switch the output on. At this stage we can not know whether the two branches represent two different sets of signaling molecules.

The NTFs-dependent outgrowth at 12h in culture is fully blocked by SU6656 - a specific inhibitor of Src family kinases (Blake et al., 2000) (Fig. 2 A). However the observed outgrowth at 40h in the absence of NTFs was not affected by SU6656 (Fig. 2 B). To confirm specificity of the SFK inhibition we took advantage of a dominant negative $pp60^{src}$ mutant (SrcDN) cloned into an adenoviral transducing construct. To allow SrcDN protein expression before the onset of axonal outgrowth, we used lower concentrations of laminin for precoating (20ng/cm^2 instead of 200ng/cm^2 in all other experiments) and quantified axonal outgrowth at later times in culture. Again increase of axonal outgrowth caused by NTFs was blocked by SrcDN but not by a control GFP-expressing adenoviral vector (Fig. 2 C). The outgrowth in the absence of NTFs was not affected by SrcDN (Fig. 2 D). These results with SrcDN and SU6656 suggest that the NTFs-dependent outgrowth is triggered by a signaling pathway that requires SFKs activation while the NTF-independent outgrowth on laminin is triggered by a different

SFK-independent signaling pathway. To verify that the two pathways are really different and independent of each other we looked for a pharmacological tool that would inhibit the NTF-independent outgrowth without affecting the NTF-activated outgrowth. We found out that roscovitine - an inhibitor of cyclin-dependent kinase 5 (Cdk5) – blocks the NTF-independent outgrowth at 40h in culture (Fig. 2 F) but does not affect the outgrowth caused by NGF, GDNF or NRTN at 12h (Fig. 2 E). In our experiments, the same concentration of roscovitine did not inhibit Erk1/2 activation as assessed by Thr202/Tyr204 phosphorylation (Fig. S2). Neither SU6656 nor roscovitine caused neuronal death under the conditions used for the axonal growth assay (Fig. S3). Taken together these results demonstrate that there are two different and independent pathways that trigger axonal growth in presence versus absence of NTFs (NGF, GDNF and NRTN) : the NTF-dependent pathway involving SFKs activation, and the NTF-independent pathway requiring Cdk5 activity. The results of inhibitory analysis (Fig. 2) allow us to describe these two pathways together as a simple Boolean network (Fig. 3 A). The network includes two inputs (laminin and NTF), Boolean operations (AND, OR) and the output – axonal growth. The generic equation for this network and the electronic scheme analog are given in Fig. 3, B and C, respectively.

In our experiments shown in Fig. 1-3 we used two different time points to differentiate the two pathways – the SFKs-dependent pathway is active at 12h in culture while the Cdk5-dependent pathway comes into play by 40h in the absence of exogenous NTFs. Cultured DRG neurons have the capacity for paracrine secretion of Brain Derived Neurotrophic Factor (BDNF) (Acheson et al., 1995). We therefore asked whether the roscovitine-sensitive outgrowth at 40h is independent of endogenous NTFs. Axonal outgrowth at 40h was not inhibited by trkB-FC chimera (Fig. 4 A). This chimeric BDNF receptor body effectively blocks the neuronal response to BDNF (Rivera et al., 2002). Axonal outgrowth at 40h was not affected significantly by blocking antibodies to GDNF and NGF (Fig. 4 A) while the same concentration of these antibodies effectively blocked outgrowth in GDNF- and NGF-treated cultures (Fig. 4 B). Propagating glia may become a source of axonal growth-promoting factors by 40h in culture. Axonal growth at 40h was not affected by the antimitotic nucleoside cytosine 1-β-D-arabinofuranoside (Fig. 4 A) indicating that this axonal growth was not stimulated by glia. We then show that the

conditioned medium collected at 40h from DRG cultures did not activate axonal growth in newly plated neurons (Fig. 4 C).

We conclude that the laminin-dependent, roscovitine-sensitive signaling pathway activates axonal growth in DRG neurons in the absence of NTFs at 40h in culture. We then asked the question: is the NTF/SFK-dependent pathway still able to activate axonal growth independently of the roscovitine-sensitive pathway at 40h? The model in Fig. 3 predicts that the NTF/SFK-dependent pathway would still activate axonal growth if the other pathway is blocked by roscovitine. Indeed roscovitine blocks axonal growth at 40h in the absence of NTFs (Fig. 5 A, B) but fails to affect outgrowth in sister cultures treated with NTFs (Fig. 5 A, C). We conclude that the NTF/SFK-activated pathway acts independently of the roscovitine-sensitive pathway. The two pathways are not additive to each other, as NTFs do not induce additional outgrowth as compared to control (Fig. 5 A). Under roscovitine treatment the NTF-activated outgrowth was further potently inhibited by SU6656 (Fig. 5 A). The outgrowth was not blocked completely in this case suggesting that there may be another NTF-activated pathway that does not involve SFKs activation.

In our experiments in Fig. 5 we applied NGF and NRTN together to activate outgrowth simultaneously in two large subpopulations of mature DRG neurons, that express TrkA and Ret receptors respectively (Fig. 6 B). These two subpopulations do not significantly overlap in adult mouse DRG (Molliver et al., 1997; Lindfors et al., 2006). Also in our experiments the values of outgrowth activated by NRTN and NGF were roughly additive to each other (Fig. 6 C). This means that each NTF (NGF or NRTN) applied separately would activate the NTF/SFK-dependent pathway in a responsive subpopulation of cultured DRG neurons. The roscovitine-sensitive pathway must be common for all neurons because roscovitine completely blocks outgrowth in the absence of NTFs at 40h (Fig. 2 E). Thus according to the model in Fig. 3 we predict that one could block the NTF-independent outgrowth with roscovitine in all neurons at 40h, and simultaneously activate the NTF/SFK-dependent outgrowth in a particular subpopulation (either TrkA-expressing or Ret-expressing) by adding NGF or NRTN separately. Indeed under Rosc treatment, NRTN and NGF applied separately induce outgrowth in responsive subpopulations (Fig. 6 A). Under NRTN+Rosc treatment outgrowth takes

place in 44% of neurons while under NGF+Rosc treatment it is 14%. Thus outgrowth in the two subpopulations together is comparable to the outgrowth in the whole untreated population – 59,4% in control, 69,5% and 66,4% under NRTN and NGF treatment, respectively (Fig. 6 A). In NGF-treated cultures combination of roscovitine and SU6656 causes complete inhibition of outgrowth. This result fits perfectly with the model in Fig. 3. Induction of small but significant outgrowth by NRTN in the presence of both inhibitors (Fig. 6 A) suggests that some other SFK-independent pathway(s) may have a minor additional role in the NRTN-activated axonal growth.

Discussion

A living cell converts information from receptor inputs into adaptive behavior by means of intracellular signaling networks (Bray, 1995). Investigation of connectivity and the operation logics of these networks is one of the key goals of modern cell biology (Sachs et al., 2002; Bhalla, 2004; Dueber et al., 2004; Sachs et al., 2005) that also has an emerging potential for drug discovery (Rajasethupathy et al., 2005). The aim of the present study was to reveal connectivity of the signaling network mediating the effects of NTFs and laminin on axonal growth, and to search for formal rules of signal propagation that would be consistent with experimental data. We demonstrate that the intracellular signaling network differentially recognizes two signaling inputs – laminin alone versus laminin + NTF by triggering two different signaling pathways.

The ability of NGF to induce axonal growth in DRG neurons was shown in classical works by Levi-Montalcini and co-authors (reviewed in (Levi-Montalcini, 1987)). An important therapeutic potential of this effect was demonstrated in the experimental model of dorsal root avulsion where NGF (as well as GDNF) promotes functional regeneration of DRG axons into the spinal cord (Ramer et al., 2000). DRG neurons also fail to develop their peripheral projections to the mammalian limb if NGF signaling is impaired (Patel et al., 2000; Tucker et al., 2001). NGF also induces outgrowth in the PC12 phaeochromocytoma cell line (Greene and Tischler, 1976), sympathetic and trigeminal neurons (Davies et al., 1981; Campenot, 1982; Arumae et al., 1993). This effect is known to involve both the Ras-Erk and PI3K-Akt signaling pathways (Markus et al., 2002). The role of SFKs in the NGF-dependent axonal growth remained unknown. Here we use SrcDN adenoviral construct and the SFKs inhibitor SU6656 to demonstrate that activation of SFKs is required for NGF-dependent axonal growth in mature DRG neurons. Understanding NGF-SFKs signaling during axonal growth in adult neurons may be important to reveal the mechanism of nerve regeneration, as both activity and protein levels of Src increase following peripheral nerve injury (Le Beau et al., 1991; Ignelzi, Jr. et al., 1992). A recent work by Tsuruda and co-authors suggests that Src may signal not only downstream, but also upstream of TrkA (Tsuruda et al., 2004). The authors demonstrate that NGF-induced phosphorylation of TrkA was

attenuated by dominant-negative Src while constitutively active Src enhanced this phosphorylation. It was demonstrated previously that the SFK activity is required for neuronal survival (Encinas et al., 2001) and axonal growth (Paveliev et al., 2004) induced by GDNF and NRTN.

Our results indicate that SFKs may be the convergence point for the signaling cascades triggered by laminin and NTFs. Interestingly Tucker and co-authors have recently come to the same conclusion using a different experimental approach (Tucker et al., 2005). Our present results further suggest that convergence of laminin- and NTFs-triggered signals on SFKs is a crucial step in the NTF-triggered axonal growth: suppression of this signaling component with SrcDN blocks the increase in axonal outgrowth caused by NRTN+NGF (Fig. 2 C). Furthermore SU6656 blocks NGF-triggered outgrowth both at 12 and at 40h in culture. Activation of SFKs is also crucial for the NRTN-dependent axonal growth at 12h in culture. The ability of NRTN to activate modest but significant outgrowth at 40h in the absence of SFKs activity is a subject for further study. We demonstrate that neither SrcDN nor SU6656 were able to prevent axonal growth on laminin in the absence of NTFs (Fig.2 B, D). We conclude that the SFKs enzymatic activity is not required for this type of outgrowth. Recent data obtained in Src/Fyn/Yes-deficient fibroblasts suggest that Src kinase activity is not necessary for the focal adhesion kinase (FAK) activatory phosphorylation (Brunton et al., 2005) but instead Src may act as an adaptor to recruit other kinases that can phosphorylate key substrates including FAK.

In our experiments all mature DRG neurons required laminin for axonal growth (Fig. S1). Laminin is known to bind $\alpha 1\beta 1$ and $\alpha 3\beta 1$ integrin receptors on DRG neurons providing the signaling input for activation of axonal growth (Tomaselli et al., 1993). Spatiotemporal regulation of laminin/integrin signaling during posttraumatic regeneration in the nervous system is still poorly understood (Silver and Miller, 2004). Developmental loss of regenerative potential is thought to be the crucial obstacle for posttraumatic regeneration of central projections by DRG neurons (Cai et al., 2001). The laminin receptor integrin $\alpha 1$ expressed in the adenoviral vector was shown to improve dramatically the ability of mature DRG neurons to regenerate axons *in vitro* both on laminin-precoated substrate and in presence of aggrecan – the condition that mimicks

glial scar (Condic, 2001). It was also shown that disruption of the laminin γ1 gene in Schwann cells leads to impaired regeneration of motor axons in sciatic nerve (Chen and Strickland, 2003). Here we demonstrate that the laminin-dependent/NTF-independent axonal growth is blocked by the Cdk5 inhibitor roscovitine. Cdk5 is known to regulate neuronal migration and axonal growth in neurons of central and peripheral nervous system (Dhavan and Tsai, 2001). Ledda and co-authors showed previously that Cdk5 mediates GDNF signaling through soluble GFRα1 receptor while GPI-linked GFRα1 signals through a different pathway (Ledda et al., 2002). It was also shown that the Cdk5-dependent pathway did not involve SFKs as it was not affected by the SFK inhibitor PP2. These data further support our conclusion that Cdk5-dependent activation of axonal growth represents a signaling pathway which is alternative to the NTF/SFK-dependent pathway for axonal growth. Laminin is also known to activate Cdk5 in cerebellar macroneurons and in a differentiated neuroblastoma cell line and in both cases this signaling pathway leads to activation of axonal growth (Paglini et al., 1998; Li et al., 2000). Little is still known about signaling pathways activated by laminin in other types of neurons (Kuhn et al., 2000). In our experiments the Cdk5-dependent pathway was not regulated by GDNF/soluble GFRα1 signaling as GDNF-blocking antibodies did not affect the roscovitine-sensitive axonal growth (Fig. 4 A).

Description of signaling pathways with conventional diagrams where arrows represent positive and negative regulations between signaling molecules are often useless for quantitative analysis, limiting the predictive and investigative value of this traditional approach (Lazebnik, 2002). Development of consistent and unambiguous rules for network representation is prerequisite to understanding bioregulatory networks (Ideker et al., 2001; Sachs et al., 2002; Kurata et al., 2003; Kohn et al., 2006). McAdams and Shapiro previously used an electrical engineering representation of Boolean networks to describe regulation of gene expression in bacteriophage lambda (McAdams and Shapiro, 1995). Using this method the network in Fig. 3 A can be presented as Fig.3 C.

In the present work we were looking for a simple and intuitive interface between formal logic and "wet" biology of cell signaling. For this purpose we have tried to avoid the mathematical details of Boolean formalism. Retrieval of precise information about the connectivity of a signaling network (i.e.: the full set of restrictions and uncertainties)

from a given set of experimental data is obviously an important further perspective for more advanced use of mathematical modeling to study protein kinase networks.

It is now evident that NGF and GDNF family ligands promote axonal growth through a pathway that involves SFKs activity. A different, SFKs-independent pathway activates axonal growth in the absence of neurotrophic factors. We believe that our findings on the operation of the signaling network triggering neuritogenesis in sensory neurons contribute to better understanding posttraumatic recovery in the nervous system, and to development of therapeutic approaches for the treatment of nerve injury.

Materials and methods

Neuronal cultures

Dissociated cultures of DRG neurons from one month old mice were prepared as previously described (Paveliev et al., 2004). One-month-old NMRI or BALB/C mice were obtained from local animal house (University of Helsinki) and sacrificed by cervical dislocation under CO_2 anaesthesia. Cells were plated (culture density 400-800 neurons/cm^2) on glass coverslips. Coverslips were pre-coated with poly-DL-ornithine (1mg/ml) overnight at +4°C and then with laminin-1 (Invitrogen, 100ng/cm^2) for 4 hours at 37°C. In some experiments axonal growth was estimated after 40 hours in culture. For those experiments a lower density (200-400 neurons/cm^2) was used. Culture medium included F12 (50%) and DMEM (50%), supplemented with glutamine, penicillin, streptomycin and serum substitute, containing 0.35% bovine serum albumin, 60 ng/ml progesterone, 16 μg/ml putrescine, 400 ng/ml L-thyroxine, 38 ng/ml sodium selenite, and 340ng/ml triiodothyronine. NGF (Promega), GDNF, NRTN (PeproTech), roscovitine and SU6656 (Calbiochem), blocking antibodies to NGF (Chemicon) and to GDNF (Amgen) were applied at the time of plating neurons.

Immunocytochemical procedures

Cultures were fixed for 15min in 4% PFA, permeabilized with 0.1% Triton X-100 and stained with rabbit polyclonal antiserum to Protein Gene Product 9.5 (PGP 9.5), (Affiniti). This marker was shown to be neuron-specific in DRG, stains axons and cell bodies of all subpopulations of DRG neurons (Wilson et al., 1988; Calzada et al., 1994). Phospho-Erk1/2$^{Thr202/Tyr204}$ antibody was from Cell Signaling. Alexa488- and Alexa350-conjugated goat anti-rabbit secondary antibodies were from Molecular Probes. Upon staining all samples were mounted in gelvatol.

SrcDN recombinant adenovirus

The SrcDN construct was a generous gift from David Kaplan (Popsueva et al., 2003). The construct co-expresses SrcDN and GFP, so neuron transduction was followed by

GFP fluorescence. DRG cultures were transduced with SrcDN or with GFP adenoviral construct when plated. In these experiments axons were visualized using primary antibodies for PGP 9.5 and Alexa350-conjugated secondary antibodies with 420nm-long pass barrier filter. In our experiments axonal growth on laminin-coated substrate was proportional to the laminin concentration. That is why we used low laminin concentrations in the experiments with adenoviruses to allow the construct expression before the onset of axonal growth (see Results).

Morphometry

The immunostained cultures were viewed using the x 20 objective (numerical aperture (NA) 0.50) of a Zeiss Axioplan 2 fluorescent microscope (Zeiss) and 300 - 500 neurons per sample were counted to measure the percentage of process-bearing neurons. Neurons with processes at least two times longer then cell body diameter were defined as axon-bearing. In all experiments statistical significance was calculated for data from at least 3 independent experiments using one-way ANOVA (Excel, Microsoft). Error bars represent standard error of mean (SEM), symbols *, ** and *** represent $P<0.05$, 0.01 and 0.001, respectively.

Image acquisition and processing

All images were collected at room temperature on gelvatol-embedded samples. Epifluorescent images were collected with a Zeiss Axioplan 2 fluorescent microscope (Zeiss) equipped with objective lenses 20x (air, NA 0.50), 40x (oil, NA 0.75) or 60x (oil, NA 1.40), AxioCam CCD camera and AxioVision acquisition software (all from Zeiss). Confocal images were collected with Leica TCS SP2 AOBS system, HCX PL APO 63x objective lens (oil, NA 1.4). 3D reconstruction was performed with Imaris software.

Online supplemental material

Fig. S1 shows that neither the NTF-dependent axonal growth at 12h in culture nor the NTF-independent growth at 40h in culture takes place without laminin precoating. Fig. S2 shows that Rosc does not inhibit phosphorylation of Erk1/2 at Thr 202/Tyr 204. Fig. 3 shows that SU6656 and Rosc do not affect survival of cultured DRG neurons.

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Abbreviations list

Cdk5, cyclin-dependent kinase 5; DRG, dorsal root ganglion; GDNF, glial cell line-derived neurotrophic factor; GFRα, GDNF family receptor α; NRTN, neurturin; NTF, neurotrophic factor; Rosc, roscovitine; SFK, Src family kinase; SrcDN, Src dominant negative; SU, SU6656.

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Figure legends

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Figure 2. **NTF-dependent and –independent types of axonal growth differ in their sensitivity to signal transduction inhibitors.** (A) The SFK inhibitor SU6656 (SU) ininhibits NTF-dependent axonal growth after 12h in culture. (B) SU6656 (2μM) does not affect axonal growth in the absence of exogenous NTFs after 40h in culture. (C)

Axonal growth induced by NGF and NRTN is blocked by Src dominant negative protein transduced in an adenoviral vector (SrcDN AV). GFP-expressing adenoviral vector (GFP AV) was used as a control. (D) SrcDN AV does not inhibit the NTF-independent type of axonal growth. In (C) and (D) low laminin concentrations (20 and 50ng/cm^2, respectively) were used for precoating. Cultures were maintained for 90h. (E) The Cdk5 inhibitor roscovitine (Rosc) (50μM) does not affect NRTN-, GDNF-and NGF-dependent outgrowth at 12h in culture. (F) Rosc (50μM) inhibits axonal growth after 40h in culture (no NTFs added). In (A, C) and (E) NTFs were applied where indicated at 100ng/ml. In (A, B, E) and (F) all NTFs and inhibitors were applied when plating neurons. In (A) significant difference between the samples treated with NTFs versus NTFs + SU is marked with asterisks. Cultures were maintained for the indicated time periods, then fixed and stained for PGP 9.5.

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Figure 4. **The roscovitine-sensitive outgrowth at 40h is not induced by endogenous NTFs.** (A) Axonal outgrowth at 40h is not inhibited by TrkB-FC chimera (200ng/ml), anti-NGF (250ng/ml) and anti-GDNF (183ng/ml) blocking antibodies, and by the glial inhibitor cytosine 1-β-D-arabinofuranoside (1μM) (AraC). (B) Blocking antibodies to GDNF and NGF block outgrowth in GDNF- and NGF-treated cultures, respectively. Anti-GDNF and anti-NGF antibodies were applied at the same concentration as in (A). (C) Conditioned medium collected from DRG cultures at 40h does not activate axonal growth in newly plated neurons.

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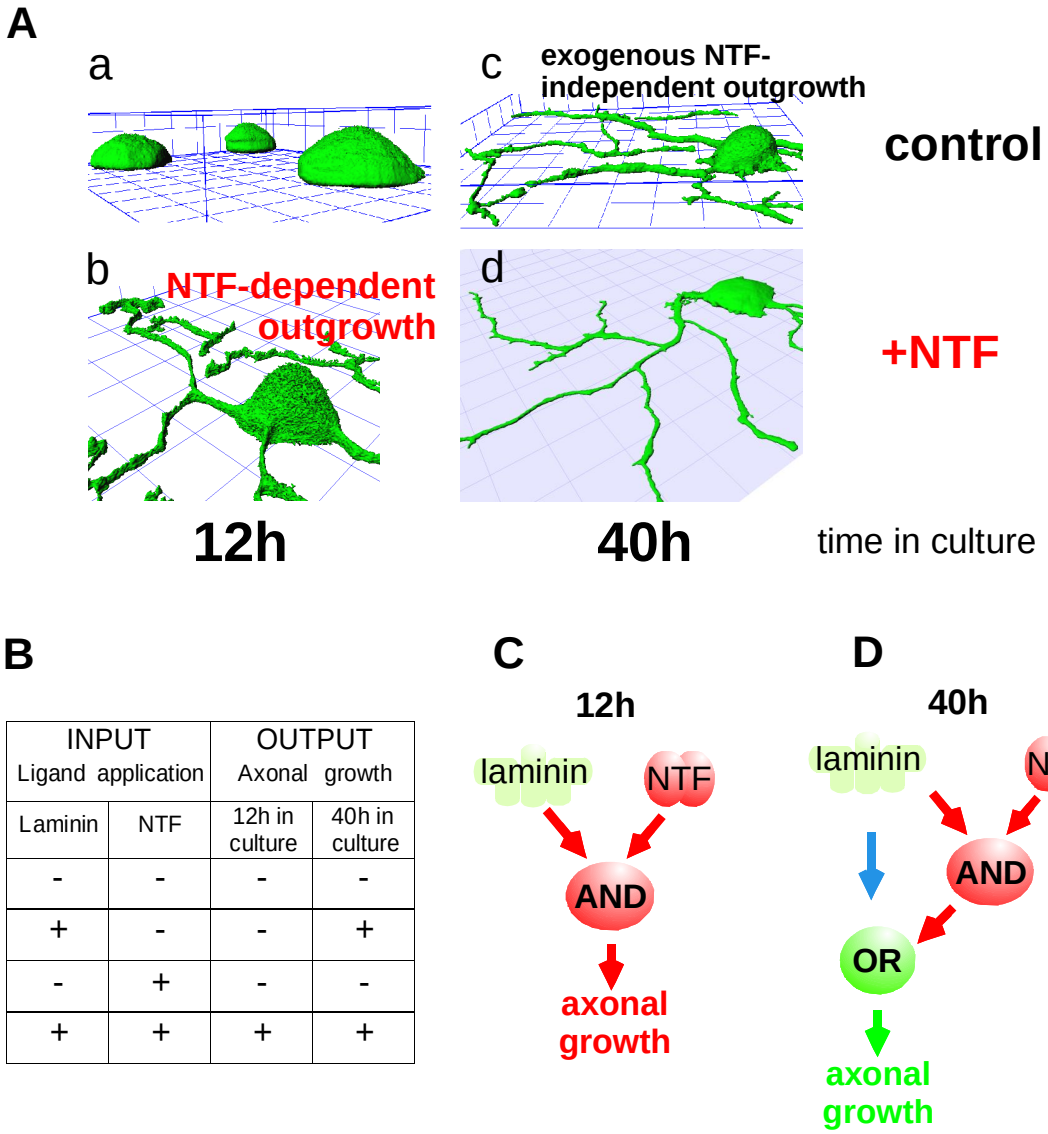
Figure S2. **Roscovitine does not inhibit phosphorylation of Erk1/2 at Thr 202/Tyr 204.** (A) NRTN and NGF (100ng/ml each) were applied for 25min at 20h in culture. Neurons were then fixed and stained for Erk1/2 phosphorylated at Thr 202/Tyr. (B) Roscovitine (50μm) was added 1h before the NTFs, otherwise conditions are the same as in (A). (C) Sister cultures that were not stimulated with the NTFs exhibit very low background activation of pErk. The scale bar is 200μm.

Figue S3. **Neither SU6656 nor roscovitine significantly affect survival of cultured DRG neurons.** The effect of SU6656 (2μm) was assessed at 5 days in culture, the effect of roscovitine (50μm) – at 40h in culure. The inhibitors were applied when plating neurons. Initial number of neurons was counted at 12h in culture. Results expressed as % of initial.



INPUT Ligand application		OUTPUT Axonal growth	
Laminin	NTF	12h in culture	40h in culture
-	-	-	-
+	-	-	+
-	+	-	-
+	+	+	+

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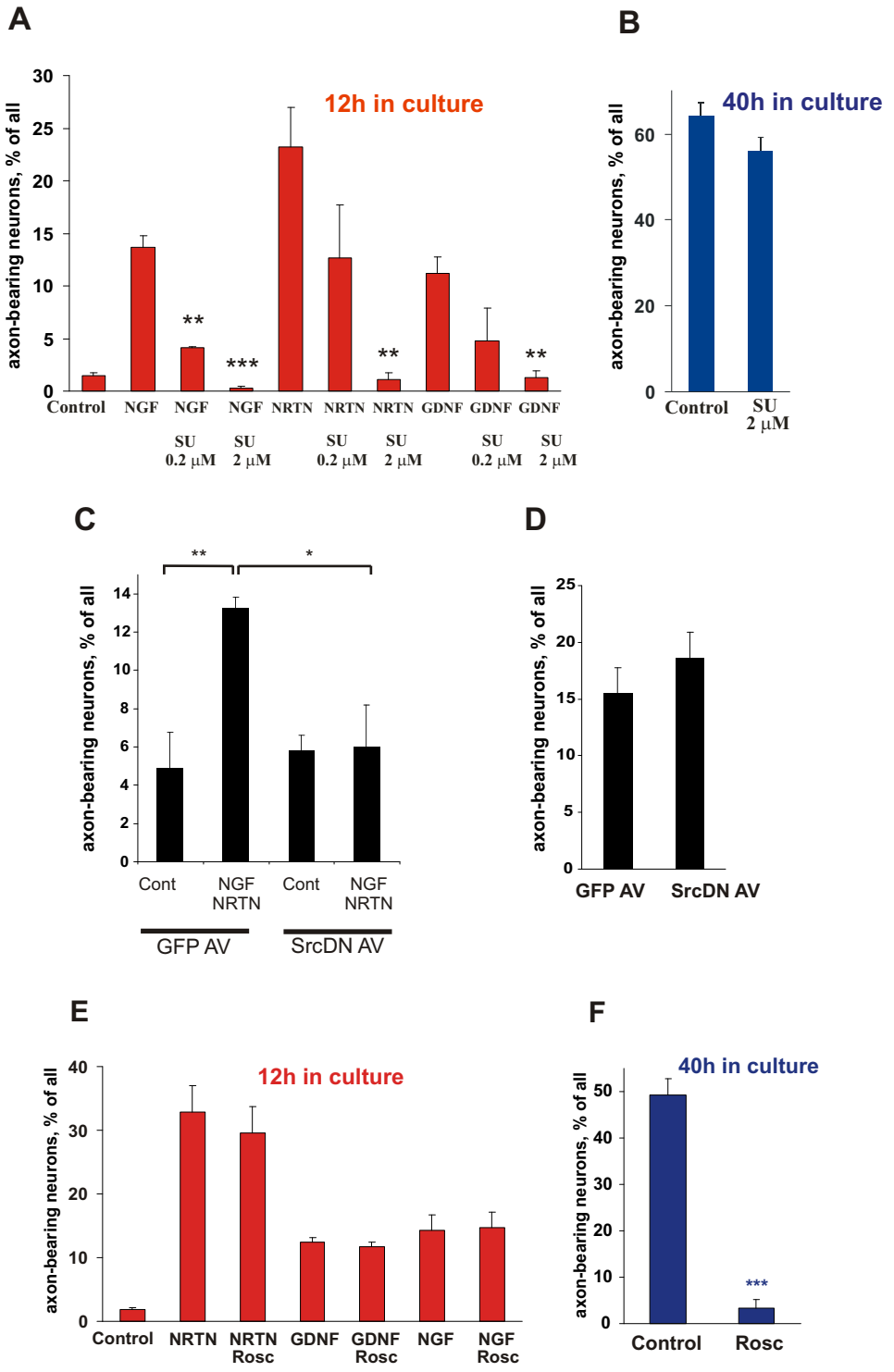

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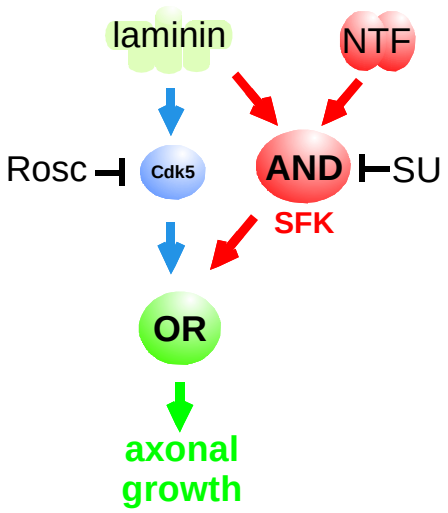
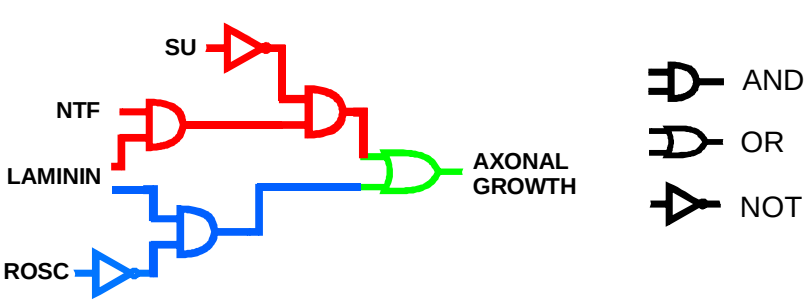
A



B

Generic Equation: $\text{Laminin} \cap (\overline{\text{Rosc}} \cup (\text{NTF} \cap \overline{\text{SU}}))$

C



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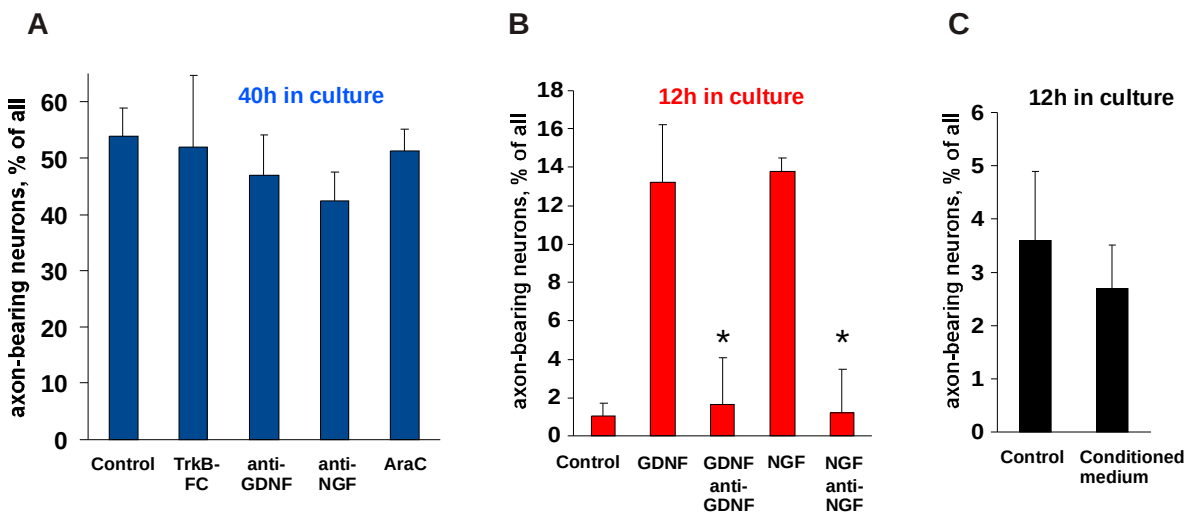

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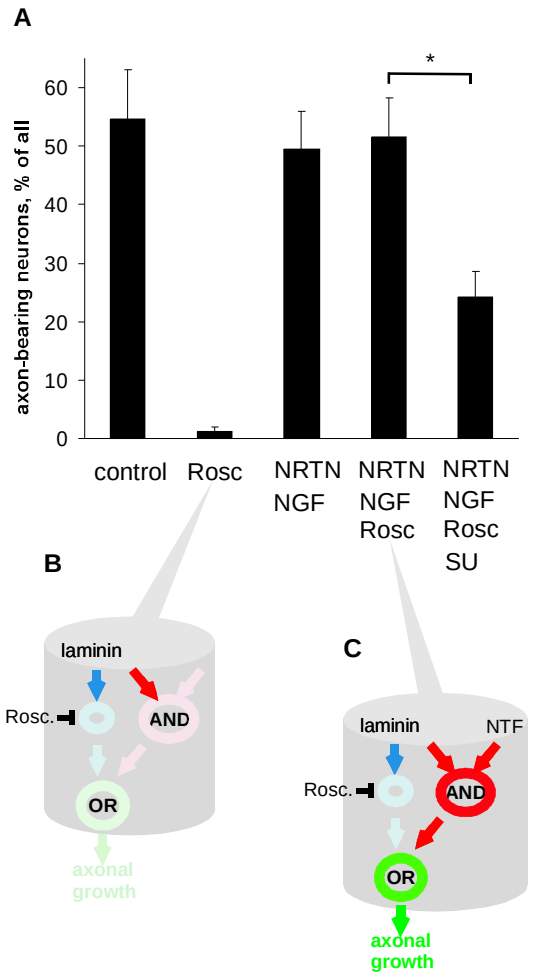

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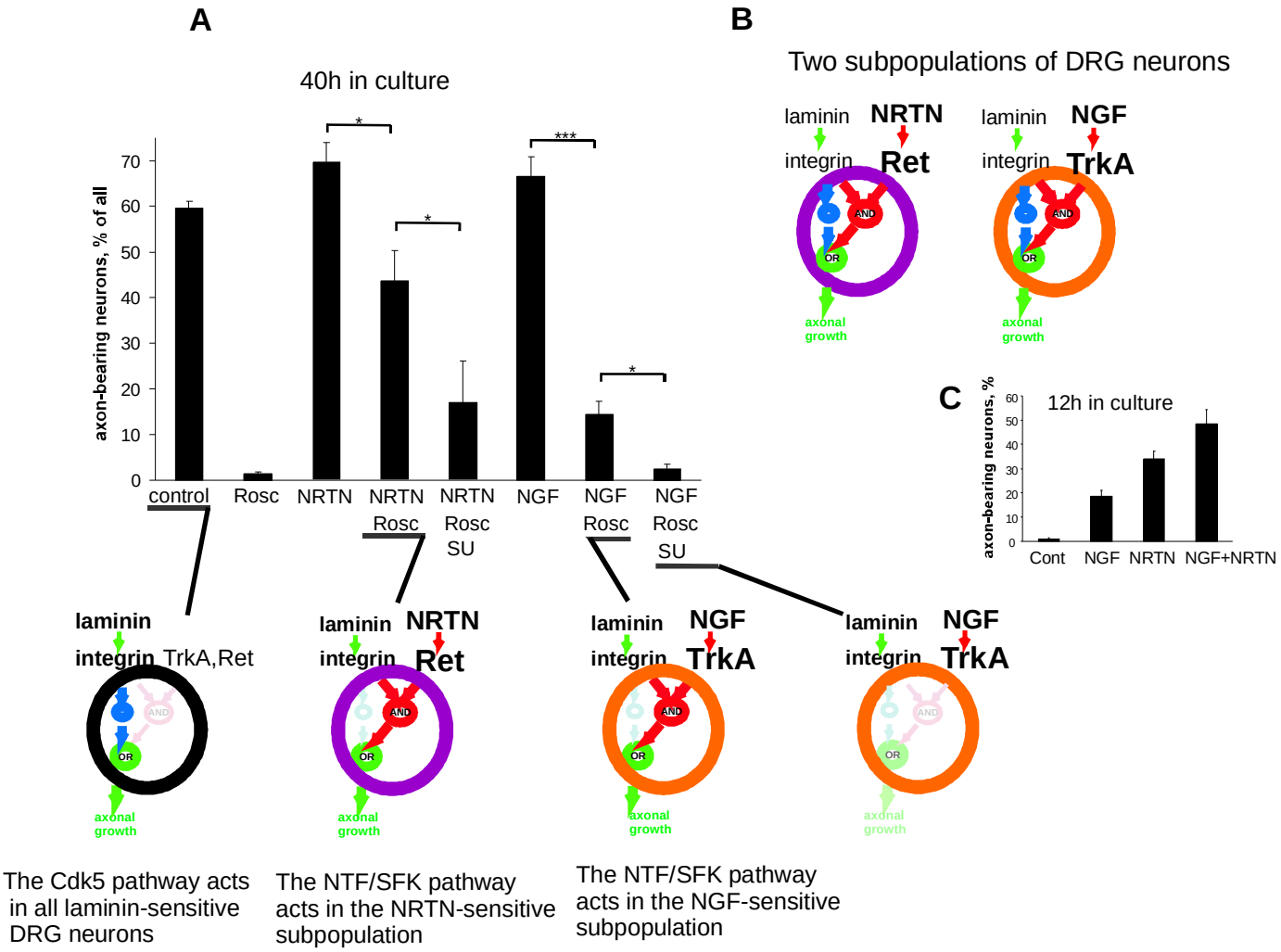

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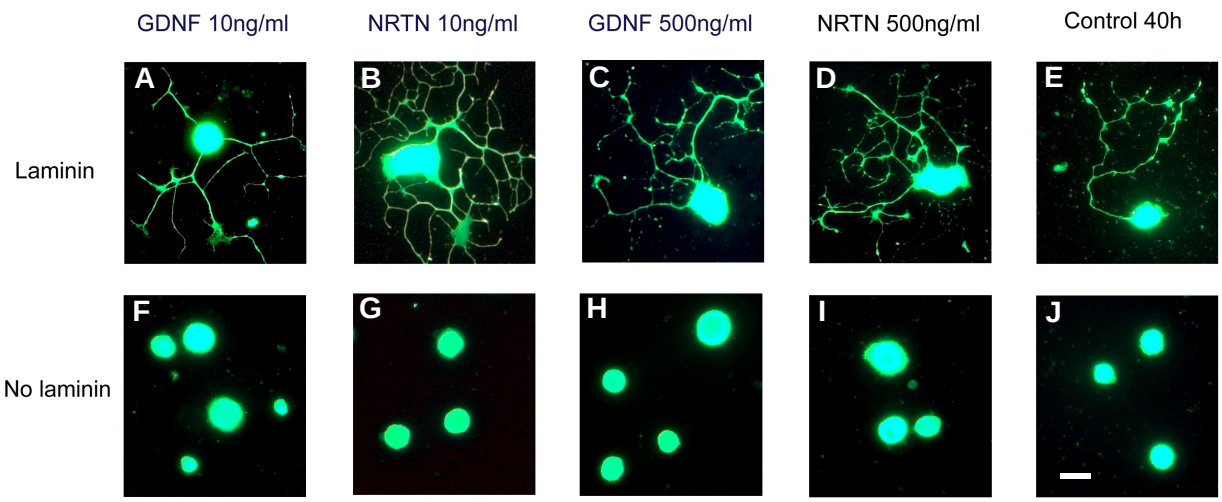

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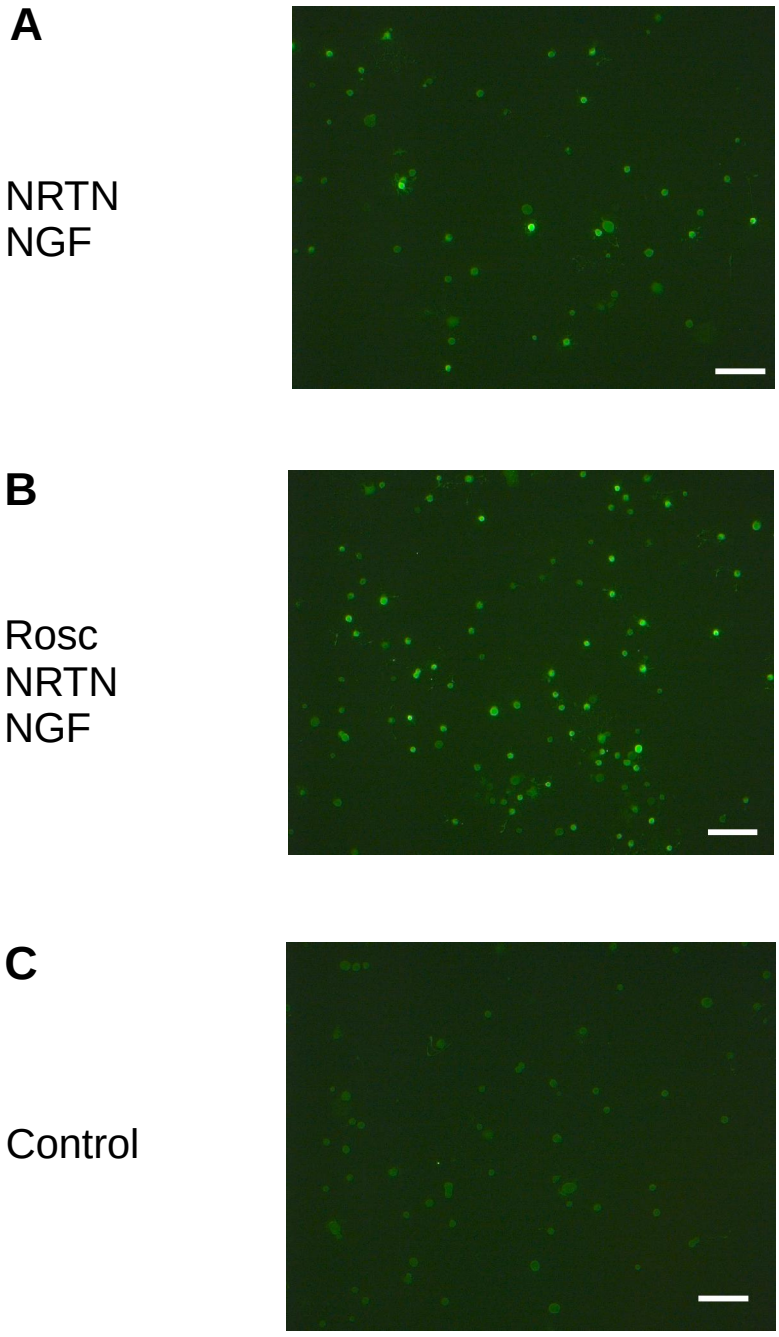
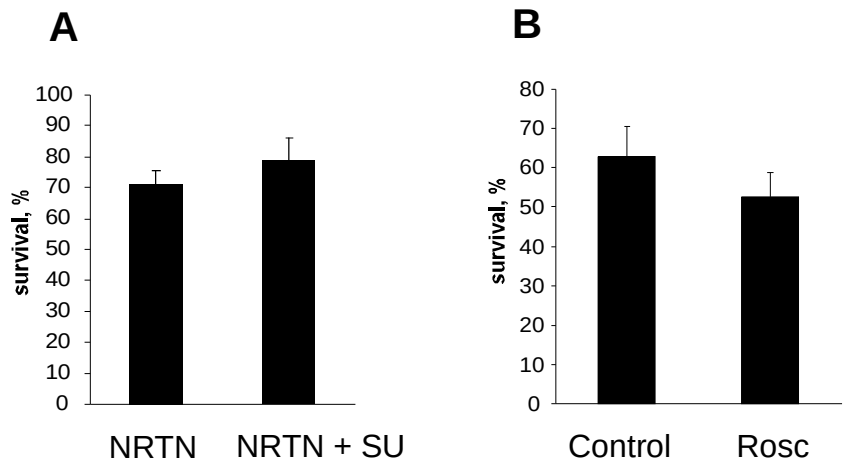


Figure S2. **Roscovitine does not inhibit phosphorylation of Erk1/2 at Thr 202/Tyr 204.** (A) NRTN and NGF (100ng/ml each) were applied for 25min at 20h in culture. Neurons were then fixed and stained for Erk1/2 phosphorylated at Thr 202/Tyr. (B) Roscovitine (50μm) was added 1h before the NTFs, otherwise conditions are the same as in (A). (C) Sister cultures that were not stimulated with the NTFs exhibit very low background activation of pErk. The scale bar is 200μm.



Figue S3. **Neither SU6656 nor roscovitine significantly affect survival of cultured DRG neurons.** The effect of SU6656 (2μm) was assessed at 5 days in culture, the effect of roscovitine (50μm) – at 40h in culure. The inhibitors were applied when plating neurons. Initial number of neurons was counted at 12h in culture. Results expressed as % of initial.