Effects of intervertebral disc cells on neural tissue

In vitro and in vivo experimental studies

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Cover illustration: A dorsal root ganglion with its neurites after 48 hours culture. © Karin Larsson

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To my Mother

ABSTRACT

Introduction: Lumbar disc herniation and sciatica are common conditions that involve interactions between intervertebral discs (IVDs) and neural tissue. The nucleus pulposus (NP) of an IVD contains at least two cell populations, notochordal cells and chondrocyte-like cells. The NP can affect nervous tissue, but the biological mechanisms behind these effects are incompletely understood. The overall aim of this thesis was to investigate the effects of the two cell populations derived from NP, notochordal cells and chondrocyte-like cells, on neural tissue in the spine.

Methods: Sprague-Dawley rats were used for both *in vitro* and *in vivo* studies. In studies I and II, dorsal root ganglia (DRGs) from newborn rats were harvested and cultured. The cells in NP were sorted by size into two cell populations, one comprising large (25-85µm), highly vacuolated notochordal cells and one comprising small (17-23µm), chondrocyte-like cells. After 24 hours culture notochordal cells and/or chondrocyte-like cells were applied to the DRG culture. After another 24 hours´ of culture neurite outgrowth was measured microscopically, using light microscopy (Study I) and electron microscopy (Study II). In Studies III and IV cell effects were evaluated using a rat disc herniation model. The cell populations were applied to L4 DRG (Study III) and to L4 DRG/nerve root (Study IV) and compared with different control systems. The analyses were performed with acute electrophysiological recordings (Study III) and with blinded light microscopic analyses (Study IV).

Results: Notochordal- and chondrocyte-like cells inhibit neurite outgrowth and reduce the diameter of neurites *in vitro* in a dose-dependent manner. Moreover, the two cell populations affect evoked neuronal thalamic activity differentially. There were pronounced neuropathological changes in both DRG and nerve roots following mechanical nerve root displacement in combination with the application of NP. The application of NP and/or the cell populations induced more discrete changes, e.g. nerve fibers with enlarged outer Schwann cell compartments.

Conclusion: The results of this thesis show that the cells in NP, notochordal cells and chondrocyte-like cells, can affect neural tissue in various ways. The findings indicate that complex mechanisms are involved in the interaction between the components of nucleus pulposus and neural tissue.

Keywords: Intervertebral disc, nerve damage, nucleus pulposus, notochordal cells, chondrocyte-like cells

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LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals (I-IV).

I. K. Larsson, E. Runesson, K. Junevik, B. Rydevik, H. Brisby. Effects of intervertebral disc cells on neurite outgrowth from dorsal root ganglion explants in culture
SPINE 2011; 36(8): 600-606

II. K. Larsson, H. Brisby, B.R. Johansson, E. Runesson, B. Rydevik. Electron microscopy analysis of neurites extending from dorsal root ganglia in vitro following exposure to intervertebral disc cells
Cells Tissues Organs, 2012;196:82-89

III. E. Nilsson, K. Larsson, B. Rydevik, H. Brisby, I. Hammar. Evoked thalamic neuronal activity following DRG application of two nucleus pulposus derived cell populations: an experimental study in rats
European Spine Journal, 2013; Jan 24, [Epub ahead of print]

IV. K. Larsson, C. Örndal, C. Nordborg, N. Sasaki, E. Runesson, H. Brisby, B. Rydevik. Neuropathological investigation of spinal nerve tissue following exposure to notochordal cells and chondrocyte-like cells: an experimental study in rats
Submitted

CONTENT

ABBREVIATIONS

DRG	Dorsal root ganglion
GAG	Glycosaminoglycan
IVD	Intervertebral disc
NP	Nucleus pulposus
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
PI	Propidium iodide

1 INTRODUCTION

Low back pain is a common condition with a lifetime prevalence of about 60-85% (Krismer and van Tulder, 2007). Low back pain leads to considerable problems and suffering for a large number of individuals, as well as high costs for the society, mainly due to disability which in turn results in a large number of lost working days. A minor part of the costs of low back pain is related to the direct cost of medical care. In the majority of persons experiencing low back pain, the underlying pathology is unknown, the duration of symptoms short and no specific treatment is required. However, in approximately 5-10% of patients experiencing low back pain, a specific cause can be determined, such as lumbar disc herniation, which often includes radiating leg pain (sciatica). In 1934, Mixter and Barr showed for the first time that sciatica was related to disc herniation (Mixter and Barr, 1934). More recently, it has been demonstrated that nerve root involvement in conjunction with disc herniation is not only caused by a mechanical nerve injury but that biochemical factors related to biological effects by nucleus pulposus (NP) on nerve roots also play an important role (Olmarker et al., 1993). The local application of NP to nerve roots, in the absence of mechanical nerve root compression, has been shown to induce changes in nerve roots, such as a reduction in nerve conduction velocity (Olmarker et al., 1993; Olmarker and Larsson, 1998; Takahashi et al., 2003), changes in behavior in mice and rats (Olmarker et al., 1998; Omarker and Myers, 1998; Olmarker et al., 2002; Yamashita et al., 2008; Otoshi et al., 2010; Sasaki et al., 2011), vascular changes (Kayama et al., 1996; Yabuki et al., 1998; Byröd et al., 2000) as well as structural changes in nerve roots (Olmarker et al., 1993; Olmarker et al., 1996; Byröd et al., 1998; Omarker and Myers, 1998). Most of these changes in nerve roots appear to be related to effects mediated by the cells in nucleus pulposus (Kayama et al., 1998; Larsson et al., 2005).

NP consists of at least two cell populations, notochordal cells and chondrocyte-like cells (Peacock, 1952; Trout et al., 1982; Trout et al., 1982). The existence of notochordal cells in NP in human adulthood is the subject of an ongoing discussion. Notochordal cells have been identified in NP up to the third decade and viable chondrocyte-like cells have been found until an age of 91 years (Peacock, 1952; Trout et al., 1982; Weiler et al., 2010). In a rabbit study, Kim et al. have shown that notochordal cells differentiate into three distinct cell types; vacuolated cells, giant cells and chondrocyte-like cells (Kim et al., 2009). In human a study, Weiler et al. have shown that cells with a chondrocyte-like morphology have a notochordal-like phenotype expressing notochordal cell markers such as cytokeratins CK-8, -18, -19, as well as galectin-3 (Weiler et al., 2010). In a number of species (e.g. rats and

non-chondrodystrophoid dogs), it has been well documented that notochordal cells are present after skeletal maturity (Hunter et al., 2004). Based on the fact that both notochordal cells and chondrocyte-like cells are present in their discs, rats are suitable animals for studies of the effect of disc cells on neural tissue.

2 BACKGROUND

Basic science and clinical observations have indicated that interaction between the intervertebral discs and neural structures plays an important part in inducing spinal pain (Olmarker et al., 1993; Kawakami et al., 1996; Olmarker et al., 2011; Sasaki et al., 2011). NP and the cells in NP have been shown to affect neural tissue both *in vitro* and *in vivo* (Kayama et al., 1998*)*. However, the relative roles played by the two cell populations, notochordal cells and chondrocyte-like cells, in the effects of NP on neural tissue are unknown. In this thesis, the aim was to clarify the roles played by the two cell populations for the reported effects on neural tissue in both *in vitro* and *in vivo* models.

2.1 Embryology

2.1.1 Development of the notochord and the spine

The notochord is commonly defined as a rod-like structure which, during the embryological period, expands in the region of intervertebral disc to form nucleus pulposus (Carlson, 2009; Bono et al., 2011). The development of the notochord starts at the end of the second week of pregnancy. At this time, it is possible to distinguish two flat layers of cells in the embryo, the epiblast and the hypoblast. Gastrulation, the process by which the three embryonic germ layers (ectoderm, mesoderm and endoderm) form from the epiblast, begins in the third week and gastrulation begins with the formation of the primitive streak. The primitive streak is an area in which cells of the epiblast form a well-defined streak. In conjunction with the appearance of the primitive streak the different axes of the embryo can be identified. An accumulation of cells, called the primitive node, is seen at the anterior end of the primitive streak. It is in the primitive node that the migrating cells are channeled into a cellular rod structure of mesenchymal cells called the notochord. The function of the primitive nodes is also to act as organizers of the future nervous system. The cells, with a diameter of about 80µm, in this slim rod become swollen with vacuoles. The notochord is important in a series of signaling inductions for transforming unspecialized embryonic cells into final tissues and organs. In particular, inductive molecule signals from the notochord stimulate the ectoderm into neural tissue and the vertebral column develops from the notochord. The cells in the notochord produce

important molecules such as noggin and chordin, known to be potent neural inducers in many species. A sonic hedgehog-mediated (an effector molecule of axial structures) induction by the notochord initiates the development of an individual vertebra. The notochord in itself becomes a segmented structure and forms the nucleus pulposus (see Section 2.4 Intervertebral discs). Mesodermal tissue on either side of the notochord and the neural tube condense to form three longitudinal columns. The medial paraxial column is one of them and it gives rise to the somites. The somites are separate blocks and within these somites there are different regions with specialized fates. One region contains cells which become the sclerotomes. These sclerotomes are the precursors of the vertebrae of the spine. There are an increasing number of cells in the peripheral portion of the disc and a decreasing number of cells adjacent to the notochord during the early stages of embryonic development. As the embryo grows (past 10 mm), the cells in the peripheral area (the annulus fibrosus) change in morphology, they become more elongated and arranged in a lamellar pattern. Collagen fibers begin to be synthesized when the embryo reaches a length of approximately 30 mm, and these collagen fibers form a collagen-rich extracellular matrix (Carlson, 2009; Bono et al., 2011). At the end of gastrulation, the ectoderm covers the embryo and gives rise to the surface ectoderm (the outer layer of the skin) and to the neuroectoderm (the entire nervous system).

2.1.2 Development of peripheral nervous system

Neurulation is a process in which a central part of the ectoderm thickens and forms a tube and this tube is called the neural tube and it forms the brain and the spinal cord. This process is induced by an interaction between the notochord and the mesoderm. When the neural tube is closed and separated from the ectoderm, a number of ectodermal cells break loose from the epithelium and migrate out. These cells are called the neural crest and they are involved in the formation of almost every part of the peripheral nervous system, such as most of the sensory and sympathetic ganglia and the Schwann cells (Alberts et al., 2008; Carlson, 2009; Bono et al., 2011).

2.2 Cells: internal organization

Eucaryotic cells, which, in contrast to prokaryotic cells, i.e. bacteria, have a nucleus, are composed of smaller elements and some of these elements are common features independent of any specialized function of the cell. Some main constituents are the nucleus with its nucleolus, which is surrounded by the cytoplasm consisting of the cytosol, the cytoskeleton and the cytoplasmic

membrane-enclosed organelles (Stevens and Lowe, 2001; Alberts et al., 2008).

2.2.1 The nucleus

The cell nucleus contains the cellular DNA, as well as the nucleolus. The nucleus is a membrane-limited compartment, formed like an envelope consisting of two membranes. The membranes have numerous pores, which help the nucleus actively or passively to transport substances between the cytosol and the nuclear lumen.

2.2.2 The cytoplasm

More than half the total cell volume constitutes the fluid matrix of the cell, called the cytosol. In the cytosol, the process of protein synthesis, protein degradation and carbohydrate metabolism takes place. Some of the intracellular compartments of the cell consist of the cytoskeleton, mitochondria, Golgi apparatus, smooth and rough endoplasmic reticulum (ER) and vesicles. The cytosol acts as a storage compartment for some metabolic products, such as glycogen and free lipids. The cytosol also contains numerous free ribosomes.

2.2.3 The cytoskeleton

The cytoskeleton of most animal cells has three types of protein filament, intermediate filaments, microtubules and actin filaments. These filaments are responsible for the organization and mechanical properties of cells. The characteristic shapes of specialized cells are created by cooperation between all these components in the cytoskeleton. Hundreds of accessory proteins link the cytoskeleton filaments to other components, as well as to each other, and without these proteins the cytoskeleton filaments would be ineffective. The cytoskeleton is also responsible for cellular polarity, i.e. gives information to the cell about possible differences within the cell between the top and bottom, or front and back. The cytoskeleton filaments are built up of small subunits, which differ in their structure. They can be either elongated and fibrous, or compact and globular. The mechanical strength is provided by the intermediate filaments, which have a diameter of approximately 10 nm and the appearance of a rope. The intermediate filaments are easy to bend but hard to break. They are made of six main type proteins, i.e. cytokeratin, desmin, glial fibrillary acidic protein (GFAP), neurofilament protein, nuclear lamins and vimentin. The position of membrane-enclosed organelles and direct intracellular transport is determined by the microtubules. Microtubules are composed of protein subunits of tubulin, alternating α-tubulin and β-

tubulin, and its shape is cylindrical. The third cytoskeleton filament is the actin filament and this filament determines the shape of the surface of the cell. Actin filament is necessary for the locomotion of the whole cell (Stevens and Lowe, 2001; Alberts et al., 2008).

2.2.4 Mitochondria

In eukaryotic cells, mitochondria occupy much of the cytoplasmic volume. Without mitochondria, the cells have to depend on anaerobic glycolysis for all their ATP (adenosine triphosphate). Various cell types have mitochondria with different morphology. Mitochondria are usually elongated cylinders and are constructed with two membranes. The mitochondria can be either mobile or remain fixed in the cytoplasm.

2.2.5 Endoplasmic reticulum and Golgi apparatus

The endoplasmic reticulum (ER) and the Golgi apparatus are two regions of an inter-communicating membrane-bound compartment which are involved in the biosynthesis and transport of cellular proteins and lipids. In inactive cells, ER is present in small amounts, in comparison with cells which synthesize and secrete protein. In the Golgi apparatus, the glycosaminoglycan chains are added to the core proteins and form proteoglycans (Stevens and Lowe, 2001).

2.3 Anatomy of the vertebral column

The vertebral column of the human spine is usually formed of 24 vertebrae. The adult vertebral column can be divided into 5 groups based on morphology and location, 7 cervical vertebrae, 12 thoracic vertebrae, 5 lumbar vertebrae, one sacral bone and one coccygeal bone. The vertebral column has several functions: weight bearing, allowing movement of the head and the trunk, and providing muscle attachments. Further, the vertebrae form the spinal canal which protects the spinal cord, from which the 31 pairs of spinal nerves exit. A vertebra consists of the vertebral body, which increases in size from C1 to L5, and the vertebral arch. The vertebral body is the main weight-bearing part. The vertebral arches form the walls of the spinal canal. This canal contains the spinal cord with its membranes, blood vessels, connective tissue, fat and the nerve roots. From the vertebral arch a number of processes extend. These processes are the single spinous process, which is a site for muscle and ligament attachment and transverse processes, which extend laterally from each side of the arch. Each vertebra has two superior and two inferior articular processes; the superior and the inferior articular processes articulate with the inferior and superior articular processes

respectively of adjacent vertebrae. These overlapping processes constitute the facet joint. Two adjacent vertebrae are connected by an intervertebral disc (IVD) and two facet joints. A human spine normally consists of 23 intervertebral discs. The vertebral column in rats has 57-60 vertebrae. They are divided into 5 groups, 7 cervical, 13 thoracic, 6 lumbar, 4 sacral and from 27 to 30 caudal vertebrae (Greene, 1955).

2.4 Intervertebral discs

The intervertebral discs (IVDs) are fibro-cartilaginous tissue between the vertebral bodies. The main function of the IVDs is mechanical, as they allow movement of the vertebral column and, since the IVDs have "shock-absorbing" properties, they are also important for the ability of spine to carry loads. The IVDs consist of three distinct regions, an outer and an inner annulus fibrosus and a centrally located nucleus pulposus (NP). The outer and inner annulus fibrosus are comprised of fibroblast-like cells and cells which resemble chondrocytes respectively. The major macromolecular components of the IVD are collagen and proteoglycans. These two macromolecules are each responsible for different biomechanical properties, such as tensile strength and resistance to compression. The collagens in the IVDs are represented mainly by two types, type I and II. The nucleus pulposus consists predominantly of type II collagen, whereas both types of collagen, type I and type II, are present in the annulus fibrosus. Collagen type II gradually changes outwards across the disc to collagen type I (Eyre and Muir, 1976; Adams et al., 1977; Eyre and Muir, 1977; Chelberg et al., 1995). The proteoglycan content is higher in the NP than in the annulus fibrosus and the most common proteoglycan in the disc is aggrecan. In a young, healthy disc, aggrecan constitutes around 70% of the dry weight of the NP and about 25% of the annulus fibrosus. The proteoglycans in the disc contain two types of sulphated glycosaminoglycan (GAG), chondroitin sulphate and keratan sulphate. These negatively charged GAGs help to attract and retain water within the matrix. With advancing age, the proportion of keratan sulphate to chondroitin sulphate increases (Adams and Muir, 1976; Adams et al., 1977; Roberts et al., 1994; Rufai et al., 1995; Urban and Roberts, 2006). The nucleus pulposus contains notochordal cells and chondrocyte-like cells/polygonal cells, i.e. there are at least two different cell populations in the nucleus pulposus. The cells in the nucleus pulposus are thought to be a remnant from the notochord (Walmsley, 1953; Minogue et al., 2010; Risbud and Shapiro, 2011). A previous study (Kayama et al., 1998) has shown that the disc cells are involved in the effects on nerve tissue. However, the roles of notochordal cells and chondrocyte-like cells in this regard are unknown.

2.4.1 Notochordal cells and chondrocyte-like cells

The notochordal cells in different species, i.e. humans, rats and pigs, contain a large number of cytoplasmic inclusions of various sizes. A dense filament network of actin can be observed around the large inclusions, in the cytoplasm and around the nucleus. A filament network is also obvious between the notochordal cells in cell clusters. The membrane around the vacuoles may have up to five distinct layers and the vacuoles may serve as osmoregulatory organelles. Some of the small inclusions in the notochordal cells are not membrane-bound. The vacuoles contain either fine granular material or material with a more coarse consistency, such as glycogen. In the chondrocyte-like cells, a thin cortex of actin around the nucleus has been observed. The chondrocyte-like cells do not contain large inclusions. Cytoplasmatic glycogen has been observed in both notochordal cells and chondrocyte-like cells (Trout et al., 1982; Trout et al., 1982; Hunter et al., 2003; Hunter et al., 2003; Hunter et al., 2004; Hunter et al., 2007). Intermediate filaments such as cytokeratin (CK8,18,19) have been demonstrated in human disc cells from embryos to adults and vimentin has been visualized in the inner annulus and in the nucleus pulposus in human cadavers discs (Götz et al., 1995; Johnson and Roberts, 2003; Rutges et al., 2010; Weiler et al., 2010), while CK8-positive cells are also found in notochordal nucleus pulposus cells from pigs (Gilson et al., 2010).

Vimentin has been shown to be more abundant in disc cells in areas that are subjected to high compressive loads, i.e. towards the disc center, as compared to disc areas subjected to less compressive loads. Cells which are immunopositive for vimentin are chondrocytic in shape (Johnson and Roberts, 2003). In a dog study, it was found that the nucleus in notochordal cells has distinct nucleoli compared with cells with a chondrocyte-like morphology. The ER and immature mitochondria have a sparse content of notochordal cells compared with chondrocyte-like cells (Hunter et al., 2003; Hunter et al., 2003). In a review article, Hunter et al discuss the protein synthesis in notochordal cells. The immature mitochondria with its close relationship to ER in the notochordal cells may explain an anaerobic metabolism (Hunter et al., 2003). The cells of the IVD generate some of their energy through anaerobic glycolysis (Holm et al., 1981; Ishihara and Urban, 1999). The low oxygen tension, 2% (hypoxia) in the IVD, reflects the IVD´s dependence on glycolysis for its ATP production. The regulation of glycolysis is mediated by hypoxia-inducible factor (HIF)-1α. HIF-1α is a transcription factor and it has been shown to be expressed by nucleus pulposus cells *in vivo* and is responsible for local oxygen tension (Rajpurohit et al., 2002; Agrawal et al., 2007). HIF-1α is also required to promote the

aggrecan synthesis. The large amount of glycogen-filled vacuoles in notochordal cells may explain their ATP production under anaerobic conditions.

It has been suggested that the large notochordal cells and small NP-cells, most likely chondrocyte-like cells, are derived from the same lineage. The notochordal cells are of notochordal origin and the small chondrocyte-like NP cells are suggested to be directly derived from notochordal cells (Minogue et al., 2010; Risbud and Shapiro, 2011). In an *in vitro* system, Kim et al studied the differentiation of rabbit intervertebral notochordal cells. They were able to observe some similarities and some differences between the notochordal and chondrocyte-like cells. Some of the similar properties were the proteoglycan production rate of the notochordal cells; the proteoglycan production rate was comparable to that of chondrocyte-like cells and both cell types expressed collagen II, aggrecan and SOX9. One difference between the two cell populations was that the chondrocyte-like cells had higher growth rates and a faster population-doubling time compared with notochordal cells. In time-lapsed cell-tracking analyses, the authors observed that the notochordal cells were able to differentiate into three morphologically distinct cell types. The three cell types were vacuolated cells, giant cells and polygonal cells. In terms of morphology, the polygonal cells were similar to chondrocyte-like cells (Kim et al., 2009).

The disc cells in the rat nucleus pulposus used in the present thesis are composed of two main populations, notochordal cells and chondrocyte-like cells/polygonal cells. The notochordal cells and the chondrocyte-like cells both have few mitochondria and sparse endoplasmic reticulum. The cytoskeleton consists of both intermediate filament, such as vimentin, and actin. Both these cell populations have glycogen-containing membrane-bound vacuoles. The vacuoles in the notochordal cells cover an area of 80-90% of the total cell area, while the vacuoles in chondrocyte-like cells cover approximately 25% of the total cell area. The main difference, as we were able to observe, between the two cell populations was the number of vacuoles. The similarity between the two populations may confirm different stages of maturation or differentiation (Fig 1 and Fig 2).

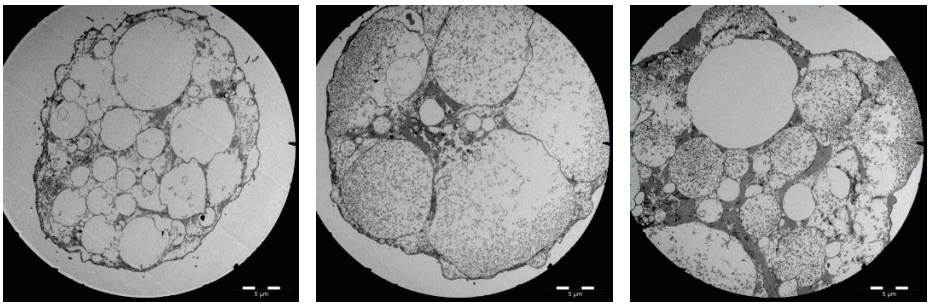

Figure 1. TEM images of notochordal cells as sorted from rat NP (Bar: 5µm).

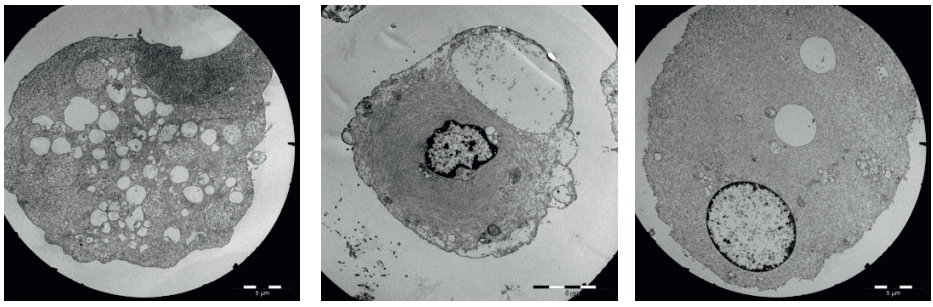

Figure 2. TEM images of chondrocyte-like cells as sorted from rat NP (Bar: 5µm).

2.5 Nervous system

The nervous system is anatomically divided into the central nervous system (CNS) and the peripheral nervous system (PNS) and it can be functionally divided into the somatic nervous system and autonomic nervous system. The spinal cord and the brain constitute the CNS (Ross and Pawlina, 2006). The PNS includes the cranial nerves, the spinal nerves, the spinal nerve roots and the peripheral nerves and they conduct impulses to or from the CNS (Dyck and Thomas, 2005). The collection of nerve cell bodies outside the CNS is called ganglia (Stevens and Lowe, 2001; Ross and Pawlina, 2006). Nerve tissue is comprised of two types of cell, nerve cells and supporting cells. In the CNS, the supporting cells are called neuroglia and, in the PNS, Schwann cells (Ross and Pawlina, 2006).

2.5.1 Neuron

The functional unit of the nervous system is called a neuron or nerve cell. These neurons are specialized to receive stimuli from other cells and to transmit electrical signals within the neuron and from the neuron to other cells, which require energy. A neuron is characterized by a cell body, axon, dendrites and a terminal button (synapse). Peripheral nerve cell bodies are collected in a ganglion, together with efferent and afferent axons. The characteristic cytology of neurons reflects high metabolic activity. The cell body contains a large nucleus with a prominent nucleolus. There is abundant rough endoplasmic reticulum and aggregates of free ribosomes (Nissl substance), which synthesize the necessary proteins. The perinuclear cytoplasm also contains a large Golgi apparatus, which produces secretory products, and large numbers of mitochondria, as energy supply. Other characteristic organelles are microtubules, lysosomes, neurofilaments, vesicles and inclusions. A neuron has only one axon and its main function is to transmit information away from the cell body to other cells or another neuron. The main function of dendrites is to obtain information from the external environment or other neurons and bring that information back to the cell body. An axon has a swollen terminal end called a synapse. The neurons communicate with other neurons and with target cells by synapses. The axonal transport system is required for intracellular communication, such as carrying information and molecules from the axonal terminal to the nerve cell body and from the nerve cell body to the axon terminal (Stevens and Lowe, 2001; Ross and Pawlina, 2006).

2.5.2 Peripheral nervous system

The peripheral nervous system includes a bundle of nerve fibers, ganglia and support cells, all held together by connecting tissue. Peripheral nerves are composed of myelinated or unmyelinated axons, Schwann cells and fibroblast, and blood vessels. The support tissues of peripheral nerves are the endoneurium, the perineurium and the epineurium (Stevens and Lowe, 2001; Dyck and Thomas, 2005; Ross and Pawlina, 2006).

Endoneurium: collagen fibrils in the endoneurium surround individual axons, Schwann cells and capillary blood vessels.

Perineurium: the perineurium surrounds groups of axons and forms small fascicles. The perineurium consists of several cell layers and collagen fibrils are present between these layers. The perineurium acts as an active diffusion barrier of metabolites that contributes to the formation of the blood-nerve barrier.

Epineurium: the epineurium binds individual nerve fascicles into a nerve trunk and consists of loose fibrocollagenous tissue. In large nerves, adipose

tissue is often included. In the epineurium, blood vessels are found and their branches penetrate through the perineurium.

The supporting cells of the PNS are the Schwann cells. They arise from the neural crest, as neurons, and are formed by the mitosis of parent Schwann cells. The Schwann cells support both myelinated and unmyelinated axons and they produce a lipid-rich insulating layer, called the myelin sheath, which surrounds the so-called myelinated axons and increases the nerve conduction velocity. The unmyelinated axons are also surrounded by Schwann cells with their external lamina. Schwann cells are involved in cleaning up PNS debris and the cells also guide the regrowth of PNS axons. The axon diameter determines the thickness of the myelin sheath at myelination. The node of Ranvier is the junction, without myelin, between two adjacent Schwann cells where Na+ channels in the axon are situated and it is the site of depolarization during nerve impulse transmission. The Schmidt-Lanterman clefts are small islands of Schwann cell cytoplasm, between the lamellae of the myelin. In the cytoplasmic clefts, there are lysosomes, mitochondria and microtubules. The diameter of the axon is correlated to the number of Schmidt-Lanterman clefts. Large axons have more clefts (Stevens and Lowe, 2001; Dyck and Thomas, 2005; Ross and Pawlina, 2006).

2.6 Cell culture

Cell culture systems have been used for many years and are a useful tool for, examining the influence of various substances on cells from the nervous system, for example. A culture can be initiated by three different methods (Schaeffer, 1990), such as organ culture, primary explant culture or cell culture. In an organ culture, the architecture characteristic of the tissue *in vivo* is retained, whereas, in a primary explant system, pieces or fragments of a tissue are used and placed on glass or plastic following attachment. In a cell culture system, the tissue or outgrowth from the primary explant is dispersed into a cell suspension (Freshney, 1994). Two major advantages of tissue culture are the control of the physiochemical environment (pH, temperature, osmotic pressure, O_2 and CO^2) and the physiological conditions (defined medium). Tissue cultures may also be exposed to a substance at a lower and defined concentration, compared with the *in vivo* situation, where 90% would be lost via secretion and distribution to tissues other than those being studied (Freshney, 1994). The cell types in tissue samples are heterogeneous compared with cell lines, which become more homogeneous after one or two passages. The phenotypic characteristics typical of the tissue, from where the cells have been isolated, are lost in a cell line. Another problem with cell lines is their genetic instability. This needs to be considered when deciding

which culture method to use in a certain experiment. The cell culture system used in this thesis is a dorsal root ganglion (DRG) explant culture. Ross G. Harrison is regarded as the founder of tissue culture. He described for the first time a developing nerve fiber in an explant culture system (Harrison, 1907).

Cell differentiation, neurite outgrowth and synaptogenesis, which can be observed using an *in vitro* neuronal system, are some of the neurodevelopmental processes that also occur *in vivo* (Radio and Mundy, 2008). The development stage of the neurons when a culture is prepared varies a great deal. The majority of intercellular processes, such as synaptogenesis and myelination, mainly occur during the postnatal phase (Gähwiler, 1988). Neurite outgrowth occurs as a consequence of both the differentiation of precursor cells and the development of lamelliopodia, which condense into processes (Craig and Banker, 1994).

Several studies have investigated the relationship between neurite outgrowth and neuron age, non-neural cells and the substrate on which they are cultured (Argiro and Johnson, 1982; Roufa et al., 1983; Argiro et al., 1984; Bray et al., 1987). Argiro et al. characterized and quantified the age dependency of several parameters of neurite outgrowth in explant cultures of cervical ganglia from embryonic, perinatal and adult rats. The differences were related to the growth onset time and the initial rate of growth. In the embryonic and perinatal explants, the onset of growth occurs within hours, while, in postnatal explants, it takes one to four days before extensions occur (Argiro and Johnson, 1982). The growth rates also differ between the perinatal and the other two groups. This growth rate difference is due to the variations in individual growth cone behavior on the collagen substrate; the perinatal rats´ growth cones translocate more rapidly on the collagen substrate compared with the other two groups (Argiro et al., 1984). DRGs attach extremely well to the collagen substrate compared with other culture surfaces (He and Baas, 2003). Windebank et al. showed that the neurite outgrowth in response to NGF is independent of anatomic position for embryonic 15-day rats´ DRG (Windebank and Blexrud, 1986).

2.7 Cell sorting with flow cytometry

Flow cytometry is a methodology which analyzes cells under flow; flow = a fluid stream, cyto = cells and metry = measuring. The technique is based on specific light scattering, such as forward scatter (FSC), which measures the relative size of the cell/particle, and side scatter (SSC), which measures the cell/particle granularity or inner complexity, and the fluorescence characteristics of single cells. A flow cytometry technique has many uses,

such as enumerating cells/particles in suspension, separating live from dead cells/particles or sorting single cells/particles for subsequent analysis. Some application areas for flow cytometry, include immunophenotyping, cytokine production and cell proliferation. Studying apoptosis, cell quantification and analyzing single cells in suspension are other applications of flow cytometry. A cytometer is a combined system of fluid stream, optics and electronics. The fluid stream introduces and focuses the cells/particles for presentation and the main function of optics is to generate and collect the light signals. The electronics convert the optical signals to proportional digital signals, process the signals and communicate with the computer.

In 1972, Leonard Herzenberg at Stanford University developed a cell sorter that separated cells stained with fluorescent antibodies. He and his coworkers coined the term "Fluorescence Activated Cell Sorter (FACS)". In 1984, the Nobel Prize in Physiology or Medicine was awarded to Niels K. Jerne, Georges J.F. Köhler and César Milstein *"for their theories concerning the specificity in development and control of the immune system and the discovery of the principle for production of monoclonal antibodies*". Their discovery of the monoclonal antibody technology enabled flow cytometry to become very successful.

2.8 Electron microscopy

Electron microscopy analyses are a basic technique in cell biology research and they are used to observe small objects in fine detail, down to a resolution of 0.1 nm, which can be compared with the human eye, which can observe objects with a resolution of 200 μm, and light microscopy, where objects can be studied down to resolution of 200 nm. In 1924, a scientist named Busch showed that a beam of electrons could be focused if it was passed through a magnetic field. The first transmission electron microscope was constructed in 1931 by Ruska and Knoll and they established the basic principles of electron optics. Ernest Ruska received the Nobel Prize in Physics in 1986. The need to study smaller structures has contributed to the continuous improvement of electron optics (Robinson and Gray, 1992). Transmission (TEM) and scanning (SEM) electron microscopy are two techniques with a different range of applications. TEM and light microscopy use the same basic principles, but there is one important difference, TEM uses electrons as a light source instead of light. Light microscopy is limited by the wavelength of light, while TEMs use a much shorter wavelength. With TEM, it is possible to observe details in the cell or different materials with a thousand times higher resolution compared with a light microscope. SEM is similar to TEM in that both instruments use a beam of electrons and electro-magnetic

coils, but in SEM, the electrons interact with the surface of the specimens (Robinson and Gray, 1992).

2.9 Electrophysiology

In order to study the functional properties of the nervous system various electrophysiological methods can be used. In general, these methods analyze the propagation of action potentials in neurons, peripherally and/or centrally. This can provide information on the functional integrity of the part of the nervous system that is under investigation. Injury to neurons or the impairment of neuronal function can be seen as changes in the electrophysiological properties of neurons, including increased excitability, as well as a reduction in the ability of neurons to propagate action potentials. These changes can occur in both the peripheral and central nervous systems (Takebayashi et al., 2001; Anzai et al., 2002; Cuellar et al., 2004).

2.10 Experimental studies of disc herniation

During the last 20 years, animal experimental research studies have provided a new insight into the area of the pathophysiology of disc herniation and sciatica. Studies of chemical and mechanical factors which might be responsible for the tissue reactions in nerve roots in association with disc herniation have been investigated by several research groups (McCarron et al., 1987; Olmarker et al., 1993; Kawakami et al., 1996; Olmarker et al., 1996; Igarashi et al., 2000; Olmarker et al., 2002; Otoshi et al., 2010; Sasaki et al., 2011). In this thesis, we have used a modified disc herniation model in rats (Olmarker et al., 1998; Omarker and Myers, 1998) in order to investigate the effect of disc cells on nerve tissue. Rats are one of the most common animals to be used for animal experiments. They have been used widely and for a long time in research and the rat physiology and function are therefore fairly well known. Rats are easy to breed and thus inexpensive. Performing surgery on rats rarely causes problems, such as infections or death. This is also important from an ethical point of view, since the number of animals can be kept to a minimum. For the studies in this thesis, the rat was a suitable animal not only because of its use in many earlier disc herniation studies, which makes it easier to compare and interpret research results, but also because of the presence of a large number of both disc cell types, notochordal cells and chondrocyte-like cells, which we wanted to study. The clinical relevance of research findings from rats can, however, be difficult to assess and a humble clinical interpretation is required.

3 THE OVERALL AIM OF THE THESIS

The overall aim of this thesis was to investigate the effects of intervertebral disc cells on neural tissue.

3.1 Specific aims

The specific aims of the studies in this thesis were:

Study I: to investigate the effects of two nucleus pulposus cell populations (notochordal and chondrocyte-like cells) on the outgrowth of neurites from dorsal root ganglia culture explants in a rat model.

Study II: to obtain further morphological information at the electron microscopy level of the regenerating neurites following the exposure of two cell populations from rat IVDs, i.e. notochordal and chondrocyte-like cells.

Study III: to study the effects on evoked neuronal activity in the rat ventral posterior lateral (VPL) nucleus of the thalamus *in vivo* following exposure to two cell populations derived from the nucleus pulposus, notochordal and chondrocyte-like cells. We also compared these results with the previously reported increase in evoked thalamic activity of NP.

Study IV: to investigate the neuropathology of DRGs and nerve roots after exposure to notochordal cells or chondrocyte-like cells in an *in vivo* disc herniation rat model.

4 MATERIALS AND METHODS

4.1 Animals

Sprague-Dawley rats (Charles River, Germany) were used to investigate the effects of intervertebral disc cells on neural tissue. Perinatal rats (1-3 days) were used for harvesting the DRGs in Study I and Study II. Sprague-Dawley rats, with a body weight of 225-250 grams were used as donor rats for allogeneic nucleus pulposus in all studies. In Studies III and IV, female Sprague-Dawley rats (225-250 grams) were used in the experimental set-ups, to investigate the neuronal effects following exposure of the intervertebral disc cells. The animal experiments were performed in sterile or clean conditions using an operating microscope. All the animal procedures were approved by the animal research ethics committee at the University of Gothenburg.

4.2 Anesthesia and surgical procedure

4.2.1 Donor rats

The NP donor rats were given an over-dose of sodium pentobarbital (60 mg/ml) (Apoteket Produktion & Laboratorier AB). NP was harvested from the tail discs, approximately 15 discs per rat, and placed in sterile culture medium, Dulbecco´s Modified Eagle Medium (D-MEM) (Invitrogen AB, Sweden) or in F12 medium (Invitrogen AB, Sweden).

4.2.2 Studies I and II

The perinatal rats were anesthetized with an intraperitoneal injection of sodium pentobarbital, followed by CO_2 inhalation and decapitation. The DRGs were exposed by a midline incision and the spinal cord was removed. The DRGs were harvested by using a 27 gauge needle.

4.2.3 Study III

Female Sprague-Dawley rats were anesthetized with an intraperitoneal induction of a mixture of fentanyl (Leptanal, 272 µg/kg, Janssen-Cilag AB, Sweden) and medetomidine hydrochloride (DomitorVet, 545 µg/kg, Orion Pharma, Finland) and anesthesia was maintained by the intermittent administration of α-chloralose intravenously (5-30 mg/kg, Rhône-Poulenc Santé, France). To limit the respiratory tract mucous secretion, atropine

(Atropin Mylan 0.5 mg/kg, Mylan AB, Sweden) was given during the preparatory dissection. Ringer Acetate and sodium buffer with glucose was administered throughout the experiment. All the animals were given pancuronium bromide intravenously (Pavulon, total dose 0.3 mg/kg, Organon, the Netherlands), to block the neuromuscular transmission, before they were tracheotomized and attached to a respirator. The heart rate was monitored via subcutaneous electrodes and the rectal temperature was maintained at 36-38°C by servo-controlled infrared lamps.

The surgical procedure was performed by transecting the left sciatic nerve at knee level. The sciatic nerve was mounted on a pair of silver hook stimulating electrodes in a paraffin pool. Paraffin was used because of its inert properties. The paraffin pool was created by skin flaps**.** The left L4 DRG was exposed and a laminectomy was performed at the Th11-12 level exposing the spinal cord and used for cord dorsum records of ascending volleys. To enable the electrode insertion into the thalamus, a craniotomy was performed (Fig. 3).

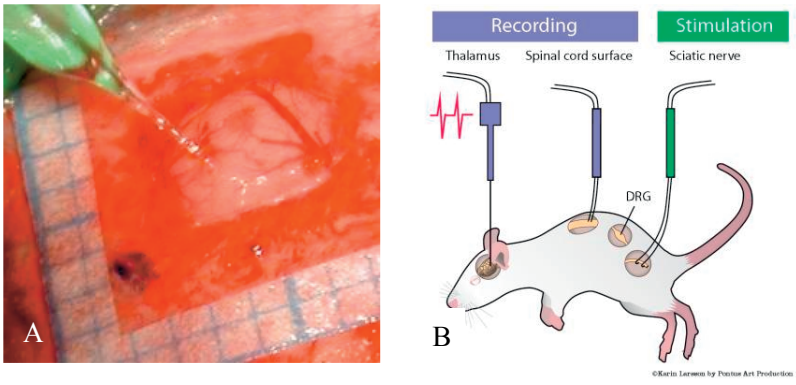

Figure 3. A. Recording electrode inserted into the thalamus. B. The positions of the recording and stimulation electrodes in the surgical setup used in Study III.

4.2.4 Study IV

The rats used in the study groups, apart from the naïve controls, were anesthetized with an Isofluran® inhalation (Baxter Medical AB, Sweden). These rats were given an intramuscular injection of Temgesic® (0.3mg/ml, Schering-Plough, Sweden) pre- and post-surgery to reduce any pain. The naïve rats were euthanized using an overdose of sodium pentobarbital.

The surgical procedure was carried out with a midline incision in the back and the left facet joint between the 4th and 5th lumbar vertebrae was removed, while the left 4th lumbar nerve and corresponding DRG were exposed.

In one of the experimental groups, the DRG/nerve root was dislocated medially by a 27 gauge needle. The needle was placed laterally to the DRG and forced gently medially. The needle was then inserted into the vertebral body and the tip of the needle was left in position for seven days, through the experiment, followed by the application of allogeneic NP (~3 mg) from one rat tail disc.

4.3 Cell separation

The cells in the NP from the donor rats were separated from each other and from the matrix by incubation with 0.1% trypsin (Invitrogen AB, Sweden) at 37°C and 95% humidity for 20 minutes. The cells were washed several times with completed DMEM (Studies I and II) or with F12 medium (Invitrogen AB, Sweden) (Studies III and IV). DMEM was supplemented with penicillin (5 µ/ml)-streptomycin (0.5%) (Invitrogen AB, Sweden), 0.1% insulin-transferrin-selenium-A-supplement (Invitrogen AB, Sweden), 0.5% bovine serum albumin (Sigma-Aldrich AB, Sweden) and 10 ng/ml nerve growth factor (NGF) (Invitrogen AB, Sweden). The cells were then filtered through a 70 µm filter just before the sorting procedure with flow cytometry.

4.4 Cell sorting by flow cytometry

The NP cells separated with trypsin (see above) were sorted using the fluorescence-activated cell-sorting technique (FACS) (FACSAria, BD Biosciences, San Jose, CA., USA) (Chen et al., 2006). In this thesis, the NP -derived cells were sorted both by their relative size, called forward scatter (FSC), and by their internal granularity and complexity, called side scatter (SSC). Cells with a different size and complexity produce different scatter patterns (Fig. 4).

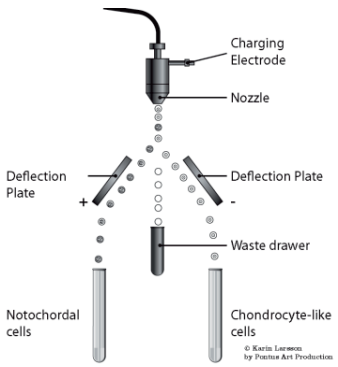

Figure 4. Schematic illustration of the basic principle of cell sorting by size.

The cells in the annulus fibrosus from rats were used as a reference population for chondrocyte-like cells to establish a gate according to FSC and SSC. For the notochordal cells, a second gate was created and a gap between the chondrocyte-like cells and the notochordal cells was positioned to avoid overlapping between the two cell populations. The gates that were determined were used for all the sorting experiments (Fig.5). The cell viability was measured using 2 µg/ml propidium iodide (PI) before the sorting procedure in all studies and also after the sorting procedure in Study II. PI is excluded by viable cells but is able to penetrate the cell membranes of dying or dead cells. The total yield of cells was determined by the sorting protocol. The mean value cells per rat tail disc were calculated.

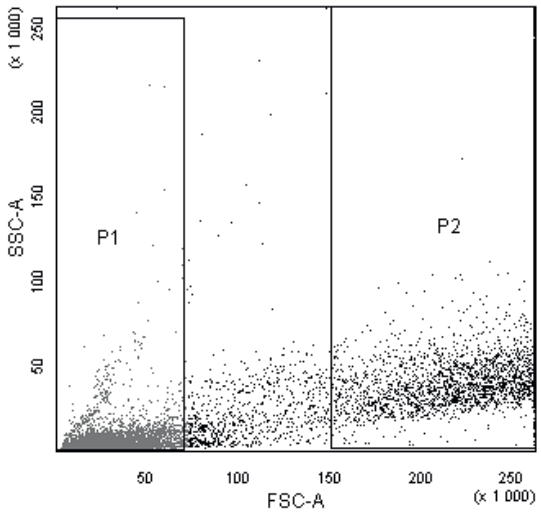

Figure 5. Flow cytometry of nucleus pulposus cells according to light scatter analysis for cell size. Y-axis indicates side scatter (SSC) and x-axis indicates forward scatter (FSC). P1 is the gate for chondrocyte-like cells and P2 is the gate for notochordal cell. The gap between P1 and P2 is positioned to avoid overlapping of the two cell populations. From Study I.

4.5 Cell culture

In Studies I and II, the DRGs were harvested from perinatal rats (see 4.2 Anesthesia and surgical procedure). The DRGs were placed on collagen (Type III, Sigma-Aldrich AB, Sweden) precoated 6-well sterile culture dishes (Fisher Scientific GTF AB, Sweden) (Study I) and on collagen precoated Termanox™ plastic cover slips and placed in a 6-well culture dish (Study II).

Five to six DRGs were placed in each well and completed DMEM (see 4.3 Cell separation) was applied to the DRGs after their adherence to the collagen surface. The cell culture dishes were then placed in a cell incubator at 37°C and 95% humidity for 24 hours´ culture. After 24 hours´ culture, two cell populations, notochordal cells and/or chondrocyte-like cells, were applied to the DRG culture alone or combined in different cell concentrations or culture medium as control. The cell concentrations are expressed as the number of cells/well. Each well contained 2 ml of medium (Table 1). The cell culture dishes were placed in the incubator for a further 24 hours´ culture.

Notochordal cells	Chondrocyte-like cells	Study
40,000	0	I
25,000	0	III, IV
20,000	0	I, II
10,000	0	I
5,000	0	I
1,000	0	I
0	150,000	III
0	40,000	I
0	25,000	III, IV
0	20,000	I
20,000	10,000	I
15,000	15,000	I
10,000	20,000	I
3,000	27,000	I
1,500	28,500	I,III
Control (medium)	Control (medium)	I,II,III,IV

Table 1. The number of notochordal and/or chondrocyte-like cells used in Studies I, II. III and IV.

4.6 Study groups

4.6.1 Study I

A total of 939 DRGs from 62 newborn rats were included in the study. Forty rats were used as donors for NP. The cells in NP were separated from each other and sorted by size, as explained above. Two cell populations were applied to the DRG culture alone or combined in different cell concentrations, after 24 hours of culture (Table 2). The cell concentrations are expressed as the number of cells/well (6 DRGs/well). The cell culture dishes were placed in the incubator for a further 24 hours´ culture before analyses. There was a variation in the evaluated DRGs in the groups due to the fact that some of the ganglia did not adhere to the culture dishes and, for each experimental set-up, one control culture dish was used (Table 2).

Group	Notochordal cells	Chondrocyte-like cells	DRGs
1	40,000	0	65
2	20,000	0	65
3	10,000	0	51
4	5,000	0	43
5	1,000	0	58
6	0	40,000	57
7	0	20,000	56
8	20,000	10,000	57
9	15,000	15,000	63
10	10,000	20,000	56
11	3,000	27,000	47
12	1,500	28,500	49
13	Control (medium)	Control (medium)	272

Table 2. Experimental groups of cells/well and the number of DRGs used for each group in Study I.

4.6.2 Study II

DRGs from 13 perinatal rats were harvested and placed on collagen-coated Termanox™ plastic cover slips. Five DRGs were placed on each cover slip (see section 4.5 Cell culture). NP was harvested from 8 rats. Three specimens from each culture condition (exposed to medium, 20,000 notochordal cells or 20,000 chondrocyte-like cells) were used to evaluate the diameter of regenerating neurites.

4.6.3 Study III

In all, 55 rats were used for acute electrophysiological experiments and as donor rats. The evoked thalamic neuronal activity was studied after ipsilateral L4 DRG exposure to notochordal and/or chondrocyte-like cells or cell suspension medium. The animals were divided into five experimental groups (Table 3). The cell concentrations are expressed as number of cells ± 10%/60 µl of F12 medium.

Group	Notochordal cells	Chondrocyte-like cells	Animals
1	25,000	0	6
2	0	25,000	6
3	0	150,000	6
4	1,500	28,500	6
5	Control (F12 medium)		7

Table 3. The number of notochordal- and/or chondrocyte-like cells used in Study III.

4.6.4 Study IV

The animals were divided into 7 different experimental groups (Table 4). The sham-operated animals were used as a control for the surgical procedure and the surgery was performed as described above without any nerve displacement or test applications. The naïve animals did not undergo any surgery. They were euthanized and the nerve root and the DRG were harvested immediately.

Study groups	Number of animals
Displacement + NP	9
NP (3 mg)	9
Notochordal cells (25,000 cells)	9
Chondrocyte-like cells (25,000 cells)	9
F12 medium (50 µl)	9
Sham	9
Naïve control	4

Table 4. The study groups and the number of animals in each group used in Study IV.

The two cell populations and F12 medium or NP were applied as shown in Figure 6.

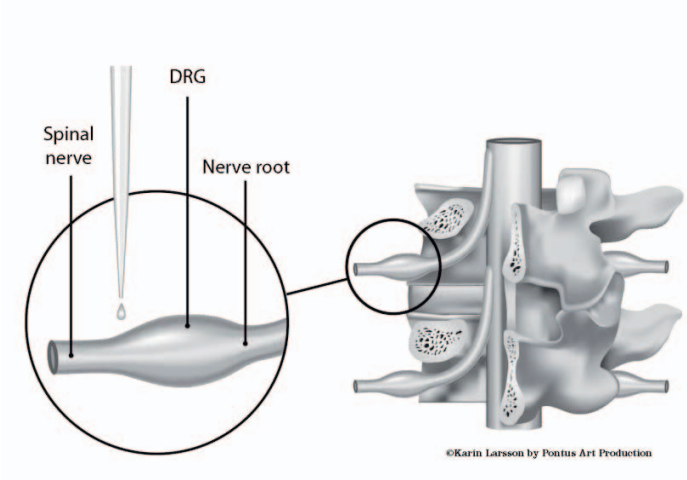

Figure 6. Schematic drawing illustrating the application site of the two cell populations and F12 or NP.

4.7 Preparation for histology

After a total time of 48 hours in culture, the DRG cultures were completed and they were fixed with modified Karnovsky fixative for 1 hour (study II). The DRG culture with its neurites was prepared for scanning (SEM) or transmission electron microscopy (TEM). In Study IV, the experiments were completed after seven days for all rats apart from the naïve group where the nerve root and the DRG were harvested immediately after the animals had been euthanized.

4.7.1 Scanning electron microscopy (SEM)

The osmium thiocarbohydrazide osmium method (OTO method) was used for all SEM preparations. This method is based on post-fixation twice with 1% osmium tetroxide (OsO_4) and with a 1% thiocarbohydrazide step. The specimens were dehydrated with an increasing concentration of ethanol, followed by hexamethyldisilazane that was allowed to evaporate and, finally, a thin film of palladium was applied. This procedure is called sputter-coating. The examination was performed in a scanning electron microscope (DSM 982, Gemini, Zeiss, Germany).

4.7.2 Transmission electron microscopy (TEM)

DRGs with regenerating neurites were post-fixed with 1% OsO_4 and 1% potassium ferrocyanide for 2 hours at +4°C, followed by *en bloc* staining with 1% uranyl acetate for 1 hour at room temperature. The samples were dehydrated with an increasing concentration of ethanol and 100% acetone and embedded in Agar 100 resin (Agar Scientific Ltd., UK). Sections of neurite extensions were made perpendicular to the culture surface and at the site of the most frequent neurite outgrowth from each DRG. The sections (60-70 nm) were counterstained with uranyl and lead before examination. The examinations were performed using a transmission electron microscope (Leo 912 AB, Zeiss, Germany) and digital images were recorded with a MegaView II CCD camera (SiS, Münster, Germany).

4.7.3 Light microscopy (LM)

After the animals were euthanized, the 4th DRG and lumbar nerve root were immediately removed and fixed in modified Karnovsky fixative for 12 hours (study IV). The specimens were post fixed in 1% OsO_4 and 1% potassium ferrocyanide for 2 hours, after which the specimens were treated with 1% uranyl acetate and dehydrated in increasing concentrations of ethanol. This procedure was followed by embedding in Agar 100 resin. The sections (0.75-1µm) of DRGs and nerve roots in Study IV were counterstained with 0.5%

azure blue and 0.5% methylene blue before light microscopy examination. The microscopic evaluations were performed blinded to the treatment.

4.8 Electrophysiological stimulation and recording

The stimulation and recording procedure was performed according to a study by Jack (Jack, 1978). The left sciatic nerve was transected, as previously described (see 4.2 Anesthesia and surgical procedure), and stimulated using 3 short stimuli to create an interval of impulses. The stimuli intensities are expressed in multiples of the threshold for the most sensitive fibers in the nerve (Jack, 1978). In the VPL nucleus of the thalamus, a glass micropipette filled with 2M NaCl was placed. The micropipette placement in the VPL nucleus was measured in mm from Bregma; posterior -2.5-3.5, lateral 3.0 and horizontal -6 (Fig.7). Bregma is the point on the surface of the skull at the junction of the coronal and sagittal sutures, and is used as a reference point for the stereotaxic procedure. In this micropipette position, low-intensity sciatic stimulation (2T) evoked scarce neuronal responses, while higher intensity stimulation (20-50T) evoked the maximum response. The maximum response corresponds to Aβ fibers and Aβ together with Aδ fibers activation, respectively but excludes the higher threshold and slower conducting C-fibers. In Study III, the neuronal responses were evoked by sciatic nerve stimulation sampled every 10 minutes in multiples series of 10-20 consecutive stimuli evaluated within a time window of 25-30 ms. Series of baseline recordings from the contralateral VPL nucleus while stimulating the ipsilateral sciatic nerve were sampled at the onset of each experiment. These recordings were used to compare the following records. After these baseline registrations, one of the cell suspensions or F12 medium was applied to the DRG and evoked thalamic activity was recorded for 40 minutes.

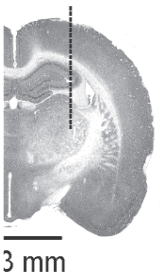

Figure 7. Representative histological section at the location of the recording site in the VPL nucleus of the thalamus marked with an electrolytic lesion. The electrode track and recording site are indicated by a dotted vertical line. From Study III.

4.9 Methodological considerations

4.9.1 DRG culture

The adhesion of DRGs or dissociated DRGs to the substrate is a prerequisite for their viability and their capacity to extend neurites. There are a large number of different methods for treating the glass or plastic culture dishes in order to promote the adhesion of DRGs. Some substrates used to coat the culture dishes are collagen, laminin, Matrigel®, poly-D-lysine and polymetylmethacrylate (Roufa et al., 1983; He and Baas, 2003; Lindwall and Kanje, 2005; Johansson et al., 2006). One important advantage of using collagen to coat the surface is the extremely good adhesion of the DRGs (He and Baas, 2003).

Different culture media are commercially available and used for tissue culture. The culture medium (completed DMEM, see 4.3 Cell separation) used in Studies I and II is based on previous studies (Larsson et al., 2005). We have used a chemically defined medium, which means excluding serum. Culture under serum-free conditions has improved the control and maintenance of cell proliferation and differentiation. The introduction of defined media supplements, insulin, transferrin and selenium, eliminates the serum requirement. Ham´s F12 medium used in Studies III and IV is based on knowledge of cartilage transplantation (Brittberg et al., 1994). In a cell culture study, using rat NP cells, Ichimura et al showed that there was no difference in growth and morphology between cells cultured in Ham`s F12 medium and those cultured in Dulbecco`s Modified Eagle Medium (DMEM) (Ichimura et al., 1991).

4.9.2 Cell separation and sorting procedure

Many different methods, such as digestion with pronase, collagenase and combinations of different substances, are used for cell separation. In this

thesis, the most suitable method for separating the cells in NP from each other and from the matrix was investigated. Digestion time and survival rate of the cells were two important factors to take into account. Digestion with 0.1% trypsin for 20 minutes was chosen (Ichimura et al., 1991). To sort the cells into different populations, we used the flow cytometric technique, sorted by size. There are descriptions in the literature of other techniques that are used for this purpose; they include nylon mesh filter separation and using culture dishes where chondrocyte-like cells adhere to the surface and notochordal cells do not attach to the surface until after 6 days (Kim et al., 2009). The latter method is not useful for the studies in this thesis, as time is an important factor in our experiments. Sorting the cells in rat NP with antibodies is not possible, since there are no specific antibodies for disc cells from rats.

4.10 Analyses

4.10.1 Study I

An inverted microscope (Nikon Eclipse TE2000-E) was used to perform the microscopic determination of the neurite outgrowth, and images were saved in a computer for further analyses. The maximum neurite outgrowth from each DRG was determined by measuring the neurite outgrowth in 4 directions, 90° from each other (NIS-Elements B, Tekno Optik AB, Sweden) (Fig.8). This was performed at 24 hours, before exposure to NP cells or medium, and at 48 hours after exposure to NP cells or medium. A total of 939 DRGs were included in the analyses. The mean of the 4 calculations was calculated and the ratio between 48 hours outgrowth and 24 hours outgrowth was calculated for each DRG. Differences were evaluated statistically using the one-way ANOVA test, followed by post hoc LSD (least significant difference) with $p < 0.05$. The SPSS statistical software package, version 16.0 (SPSS Inc., Chicago, IL, USA), was used for the statistical calculations. The measurement was performed by one person blinded to the treatment of the DRGs. Additionally, 52 and 58 DRGs were measured to conduct inter- and intraobserver analysis. Using the known measured values for 1,000, 5,000, 10,000 and 20,000 of notochordal cells, the approximated value for intermediate concentration was calculated by assumption of a linear relationship.

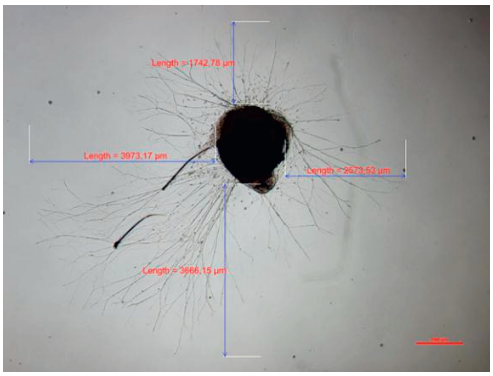

Figure 8. DRG after 48 hours in culture. The maximal outgrowth was determined by measuring the neurite outgrowth in four directions, 90° from each other.

4.10.2Study II

To perform morphometric analyses of regenerating neurite diameter, 3 specimens from each culture condition (DRG culture exposed to medium, notochordal cells or chondrocyte-like cells) were sectioned at a distance of 100 µm from the DRG body (Fig. 9). The diameter of neurites perpendicular to the culture surface (thus avoiding the influence on the size of the angle between the radiating cellular extensions and the section) was measured and recorded using the EsiVision software package.

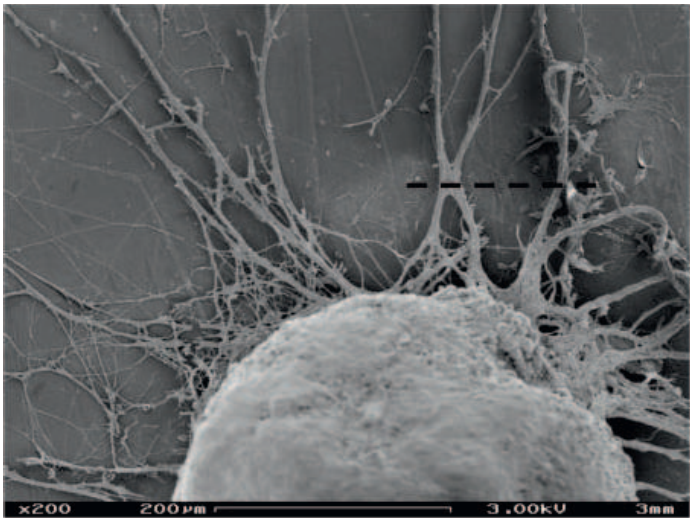

Figure 9. SEM image showing the position where transverse sections (indicated by dashed line) were performed at a distance of 100 µm from the DRG, in order to evaluate the diameter of regenerating neurites. From Study II.

Data from transected regenerated neurite diameter exposed to medium (n=575), chondrocyte-like cells (n=419) or to notochordal cells (n=1,009) were compiled. Measurements of the diameter of regenerating neurites from three DRGs of each condition were pooled and compared statistically and evaluated using the one-way ANOVA test, assuming independent observations. The one-way ANOVA was followed by post hoc LSD, with a significance level of $p < 0.05$. The SPSS statistical software package, version 16.0, was used for the statistical calculations.

4.10.3 Study III

A software system (designed by E. Eide, T. Holmström and N. Pihlgren, University of Gothenburg) was used to store the original data records from the electrophysiological experiments, as well as the averages of 10-20 consecutive records. The mean number of evoked baseline responses was set at 100% and data from each series were presented as a percentage ±SEM of the initial values. To compare the number of responses evoked at different time points between groups, the Kruskal-Wallis test and paired t-test were used to compare changes in the number of evoked responses between time points in the individual groups with a significance level of $p < 0.05$. The SPSS statistical software package, version 17.0, was used for the statistical calculations.

4.10.4 Study IV

The naïve animals were used to determine morphology baseline for evaluation of nerve damage in the study groups. After establishment of the morphology baseline the examinations were blinded to the treatment for the whole study, except for animals undergoing the combined treatment, i.e. nerve displacement and NP application. According to previous studies the combined treatment has been shown to cause severe nerve fiber damage, and this group was therefore used as a positive control (Omarker and Myers, 1998). The assessment of histological nerve fiber damage was restricted to myelinated nerve fibers. Unmyelinated nerve fibers may not be analyzed by light microscopy. The morphology was evaluated regarding the proportion of fragmented nerve fibers with debris in axon and/or Schwann cell, distended myelin sheaths with or without detectable axon, denuded axons and loss of myelinated fibers. It is not possible to differentiate between demyelination and axonal degeneration using light microscopy examination. The sections were analyzed based on two different morphology parameters; 1. Percentage of damaged nerve area in relation to the total cross-sectional area and 2. Percentage of damaged axons in the affected area. Moreover, the Schwann

cells were investigated with regard to Schwann cells with an enlarged outer compartment (Table 5).

Percentage of damaged nerve area in relation to the total cross-sectional area	1-25	26-50	51-75	76-100
Percentage of damaged axons in the affected area	1-25	26-50	51-75	76-100
Number of Schwann cells with an enlarged outer compartment	0	+ =sporadic	++ =modest	+++ =numerous
Nominal values	1	2	3	4

Table 5. The criteria used for the light-microscopic examination and the nominal values used for these criteria. For details, see text. From Study IV.

The two nerve damage criteria and the histological assessment of the number of Schwann cells with an enlarged outer compartment were converted into nominal values. The different percentage group nominal values were as follows; 1-25%=1, 26-50%=2, 51-75%=3 and 76-100%=4 and, for the Schwann cell assessments, the number of Schwann cells with an enlarged compartment was converted to nominal values as 0=1, +=2, ++=3, +++=4 (see Table 5). The statistical analyses were performed by comparing and evaluating the nominal values using one-way ANOVA test, followed by post hoc LSD with a significance level of $p < 0.05$.

5 RESULTS

5.1 Study I

About 85% of the harvested DRGs adhered to the bottom of the precoated cell culture dishes. Adherence to the bottom is a prerequisite for neurite outgrowth. Different cell populations were applied to the DRGs and an average of 94% of all DRGs demonstrated an increased neurite outgrowth (Table 6). The viability was determined before the sorting procedure and the viability of the total disc cell population was approximately 94%. The larger notochordal (25-85 µm) cells showed more pronounced cell death with a mean viability of 75%. The smaller chondrocyte-like cells (17-23 µm) had a viability of 97%. It was possible to determine an approximate number of cells in one rat tail disc with a FACSAria instrument and the cell number was 6,000 notochordal cells and 50,000 chondrocyte-like cells.

Group	Notochordal cells	Chondrocyte-like cells	Percent of ganglia with increased outgrowth
1	40,000	0	92%
2	20,000	0	89%
3	10,000	0	94%
4	5,000	0	93%
5	1,000	0	98%
6	0	40,000	91%
7	0	20,000	95%
8	20,000	10,000	95%
9	15,000	15,000	98%
10	10,000	20,000	91%
11	3,000	27,000	96%
12	1,500	28,500	88%
13	Control (medium)	Control (medium)	96%

Table 6. The experimental groups, the number of cells/well and the percentage of DRGs with increased outgrowth. From Study I.

5.1.1 Notochordal cells

A statistically significant inhibition in neurite outgrowth compared with medium was induced when a cell concentration of 40,000, 20,000 and 10,000 notochordal cells/well was applied to the DRGs. A cell concentration of 5,000 and 1,000 cells/well of notochordal cells did not inhibit the neurite outgrowth compared with medium (Fig. 10; from Study I).

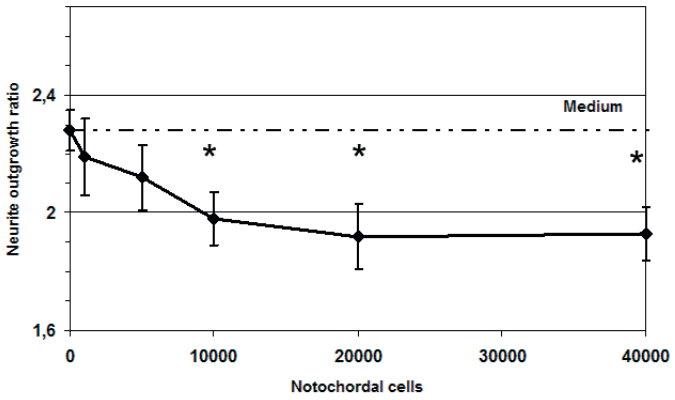

Figure 10. 10,000, 20,000 and 40,000 notochordal cells/well induced a statistically significant reduction in the ratio of neurite outgrowth compared with medium.

5.1.2 Chondrocyte-like cells

The neurite outgrowth was statistically inhibited when chondrocyte-like cells in a cell concentration of 40,000 cells/well were applied. However, chondrocyte-like cells in a lower concentration (20,000 cells/well) did not affect the neurite outgrowth (Fig. 11).

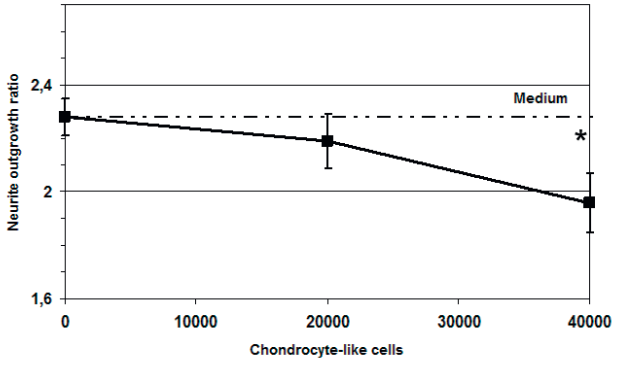

Figure 11. The effect of the chondrocyte-like cells in a cell concentration of 40,000 cells/well on neurite outgrowth was statistically significant. From Study I.

5.1.3 Cell combination groups

When cells were applied in a combination of notochordal cells (1,500 cells/well) and chondrocyte-like cells (28,500 cells/well), the neurite outgrowth was statistically significantly inhibited compared with medium application. None of the other combinations of cells (notochordal cells and chondrocyte-like cells) resulted in this kind of effect compared with medium (Fig.12).

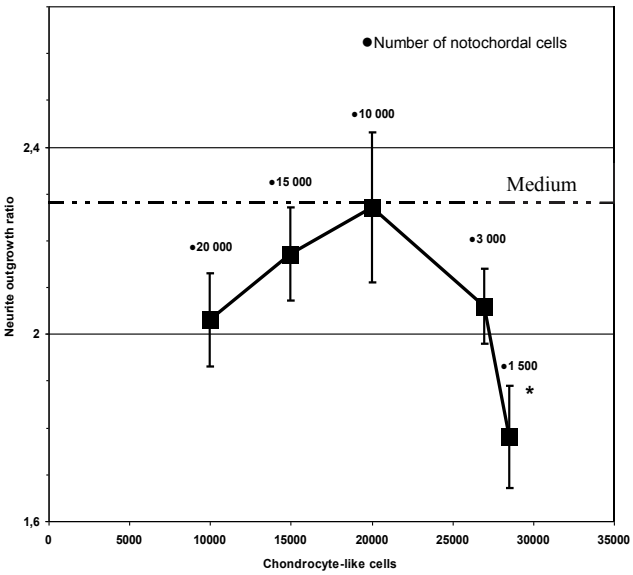

Figure 12. A combination of chondrocyte-like cells and notochordal cells appears to have an interactive response. From Study I.

5.1.4 Inter- and intra-observer accuracy

The inter- and intra-observer accuracies of the neurite outgrowth were calculated. The inter-observer accuracy was 93% and the intra-observer accuracy was as 96%.

5.2 Study II

After the sorting procedure, the viability decreased by approximately 0.6% for the notochordal cells compared with before the sorting and for the chondrocyte-like cells, where viability decreased by 0.7%.

5.2.1 Descriptive SEM analyses of neurites

From cultured DRGs, radiating neurite outgrowth was seen as bundles of varying size and complexity by SEM (Fig. 13). Individual nerve cell processes often branched and formed flattened anchoring extensions to the substrate, richly equipped with filopodia; this was seen in the distal and leading end of the extensions (Fig. 14). On the collagen surface, close to the DRG, flattened cells (interpreted as fibroblasts) formed a semi-continuous layer traversed by the neurite bundles (Fig. 15). The neurite outgrowth extended past the fibroblast zone and sometimes the fibroblasts formed contacts with the neurite bundles and partly covered them. It was not possible to identify Schwann cells with certainty using SEM analyses. After exposing the neurite outgrowth to notochordal cells or chondrocyte-like cells, no morphological changes in neurites or supporting cells were observed.

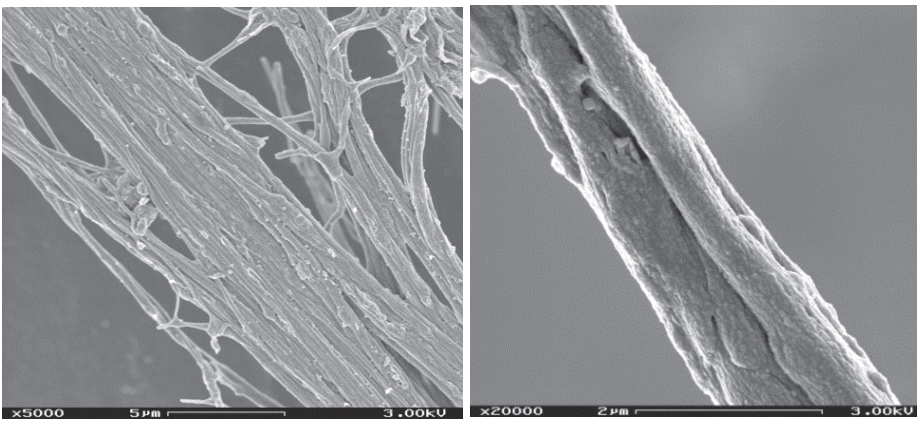

Figure 13. SEM images of radiating neurites from a cultured DRG.

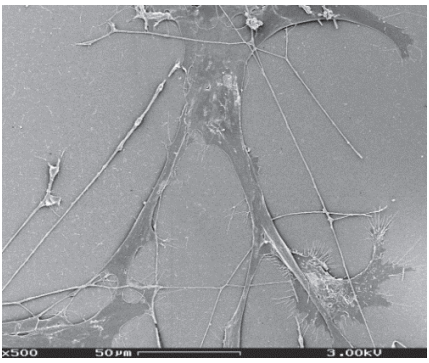

Figure 14. SEM image of flattened anchoring extensions to the substrate from individual nerve cell processes.

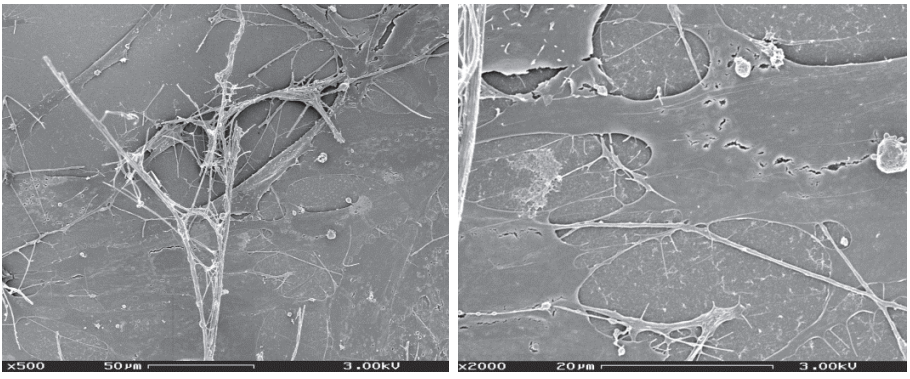

Figure 15. SEM images of fibroblasts which form a semi-continuous layer.

5.2.2 Descriptive TEM analyses of neurites

It was confirmed with TEM on transverse sectioned neurite bundles that the bundles were mostly formed by individual, tightly adhering and uniform neurites with the expected cytoplasmic content of neurofilaments, microtubules and single groups of vesicles (Fig. 16A). In some larger bundles of neurites, a cytoplasm-rich large cell showed a complex interaction with the neurites by extending several cytoplasmic processes seemingly subdivided the neurite bundle into smaller compartments (Fig. 16B).This organization may also reflect Schwann cell-to-neurite interactions. Between the neurite bundles and the culture substratum, a large number of flattened cells with a probable fibroblast identity were observed (Fig. 17). A scattered single neurite had become enclosed by another cell, in a manner strongly indicating an early Schwann cell investment of the neural structure (Fig. 18) The TEM-based description of cellular organization and interaction did not differ to any noteworthy degree between the three culture conditions.

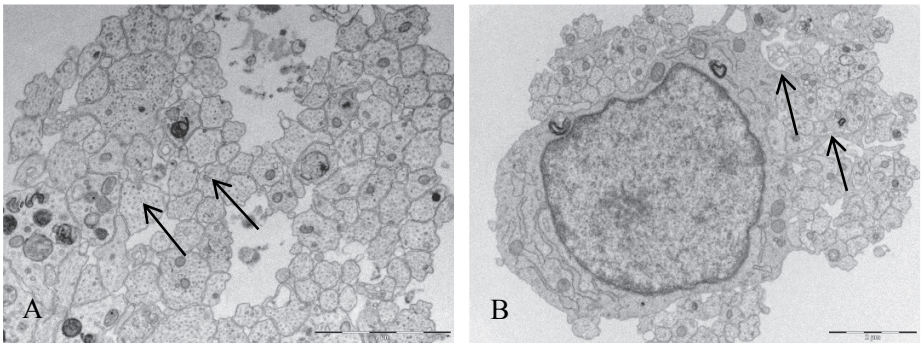

Figure 16. TEM: Transverse section of neurites. A. Neurites with neurofilaments and microtubule (arrows). B. A cell sending out numerous cytoplasmic processes that apparently divide the neurite bundle into smaller compartments (arrows) (Bar: 2µm).

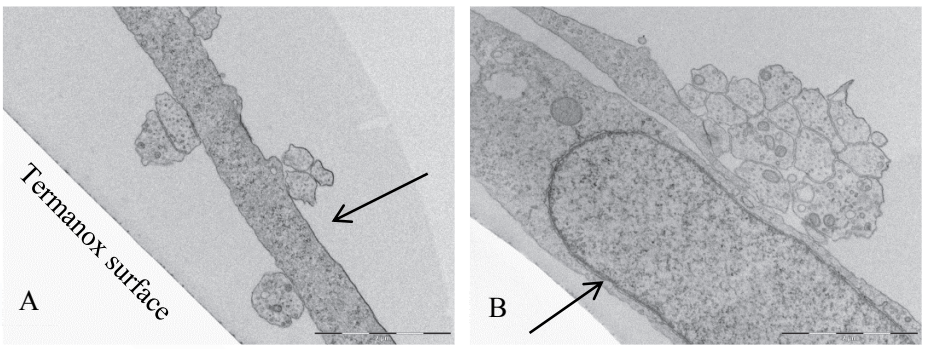

Figure 17. A and B. TEM images of a fibroblast closely adhering to neurite bundles (arrows) (Bar: 2µm). Figure 17B is from Study II.

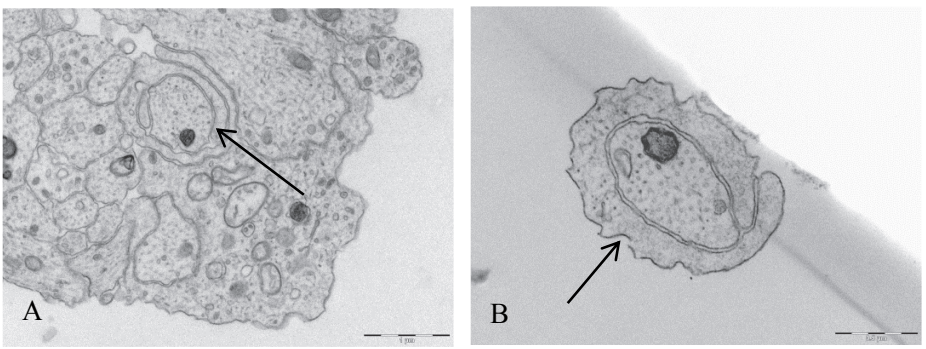

Figure 18. TEM images of a single neurite enclosed by another cell, strongly indicating a Schwann cell (arrows) (Bar: A: 1µm and B: 0.5µm). Figure 18B is from Study II.

5.2.3 Calculation of neurite diameter on transverse sections by TEM

There was a statistically significant reduction in neurite diameter when notochordal cells (20,000 cells/well) were applied to the DRG and neurites compared with the cultures exposed to culture medium (control group) and with chondrocyte-like cells (20,000 cells/well) (Fig. 19). There was no statistical significant difference in neurite diameter between DRG cultures exposed to chondrocyte-like cells compared with DRG cultures exposed to culture medium.

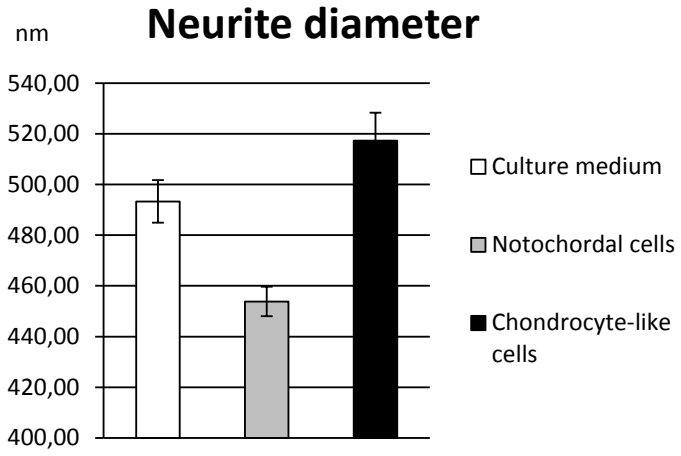

Figure 19. Mean value (±SEM) of neurite diameter value from the three experimental groups. Notochordal cells induced a significant reduction of the neurite diameter compared with medium ($p<0.0001$) and chondrocyte-like cells ($p<0.0001$), when applied to DRG and neurites. From Study II.

5.3 Study III

In all the experiments, the cell viability was above 80% (the mean viability for the notochordal cells was 84%, while it was 97% for the chondrocyte-like cells). The cell suspension medium did not induce any change in evoked thalamic activity during 40 minutes of recording (Fig. 20).

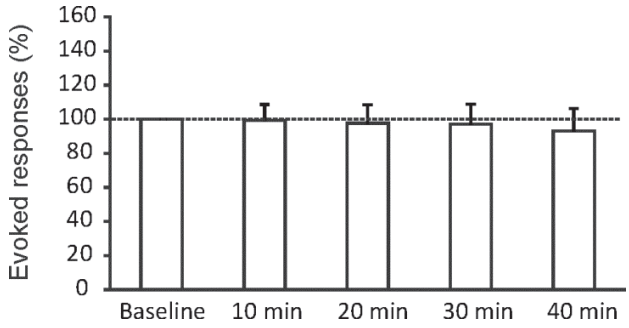

Figure 20. Control experiment. Mean number of evoked responses in VPL nucleus in percent of baseline during 40 minutes of recording with F12 medium. From Study III.

5.3.1 25,000 notochordal cells and 25,000 chondrocyte-like cells

The result after the application of 25,000 notochordal cells on the DRG was a statistically significant decrease in evoked thalamic activity within 10 minutes. This decreased activity lasted for 40 minutes, i.e. the recording period (Fig. 21a). However, the application of 25,000 chondrocyte-like cells did not evoke any statistically significant changes in thalamic activity during the same period as that for the notochordal cells (Fig. 21b). There was a statistically significant difference between notochordal and chondrocyte-like cell populations when comparing the mean number of evoked thalamic responses after 40 minutes (Fig. 21c).

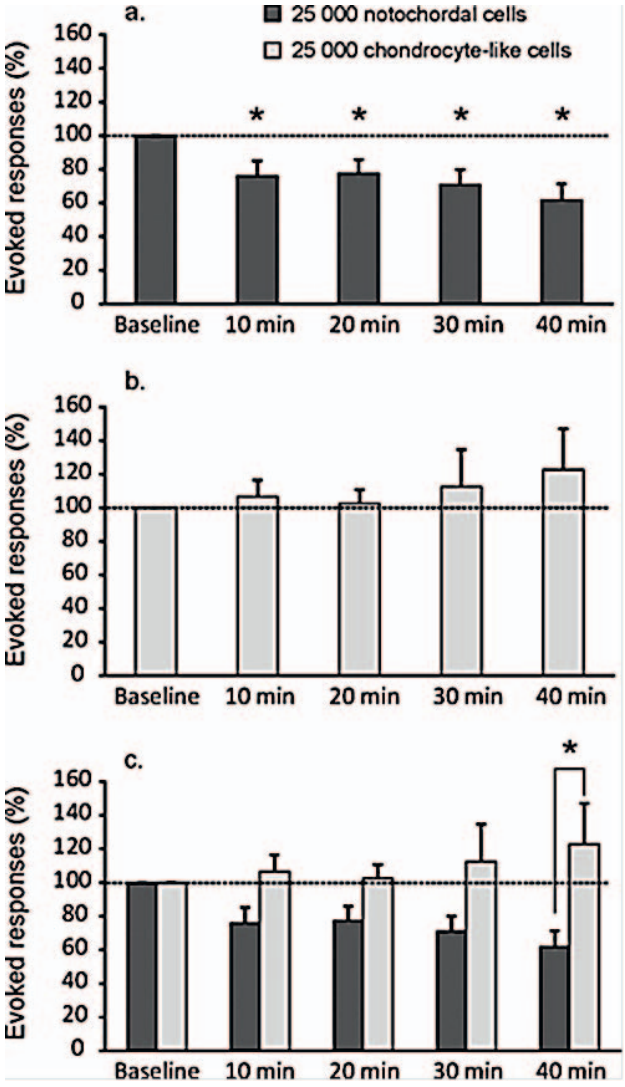

*Figure 21. Mean number of evoked responses in the VPL nucleus of the thalamus following the application of 25,000 notochordal or chondrocyte-like cells on the DRG. Averaged data during 40 minutes of recordings with a) notochordal cells (n=6), b) chondrocyte-like cells (n=6) and c) both groups presented in the same graph for comparison. * $p < 0.05$, ± SEM. From Study III.*

5.3.2 150,000 chondrocyte-like cells

150,000 chondrocyte-like cells did not result in any changes in evoked thalamic activity compared with baseline during a time period of 40 minutes

(Fig. 22), similar to the observation with smaller number of chondrocyte-like cells. Further, there were no statistically significant differences in evoked thalamic responses at any time point compared with the results obtained when DRGs were exposed to 25,000 chondrocyte-like cells.

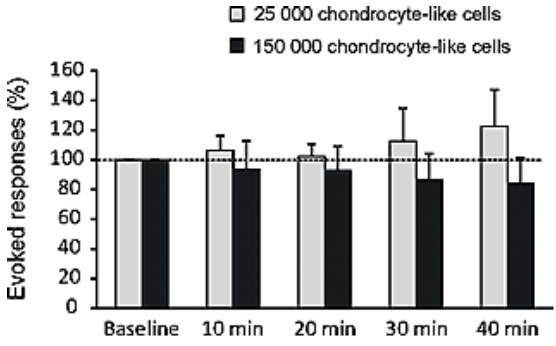

Figure 22. Mean number of evoked responses following the application two of different numbers of chondrocyte-like cells. Average number of responses during a period of 40 minutes after 25,000 (n=6) or 150,000 (n= 6) chondrocyte-like cells. From Study III.

5.3.3 Combination of notochordal cells and chondrocyte-like cells

The application of a total number of 30,000 cells in a combination containing 1,500 notochordal cells and 28,500 chondrocyte-like cells (ratio 1:20) did not induce any changes in evoked thalamic activity during 40 minutes of recording (Fig. 23).

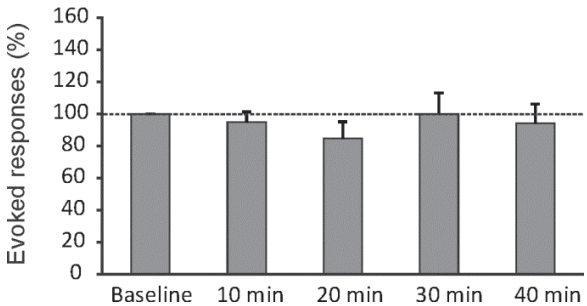

Figure 23. Effects following the application of a combination of cells. Average number of evoked responses during 40 min of recordings after the application of 30,000 cells in a combination of notochordal and chondrocyte-like cells (n=6). Data presented as ±SEM. From Study III.

5.4 Study IV

The viability of notochordal cells and chondrocyte-like cells was 90% and 96% respectively.

5.4.1 Nerve fiber damage

The spinal nerves and dorsal root ganglia exhibited severe nerve fiber damage seven days after nerve root displacement and the application of NP. In this group, the element of denuded axons was more pronounced and epineural granulation was seen in all animals (Fig. 24).

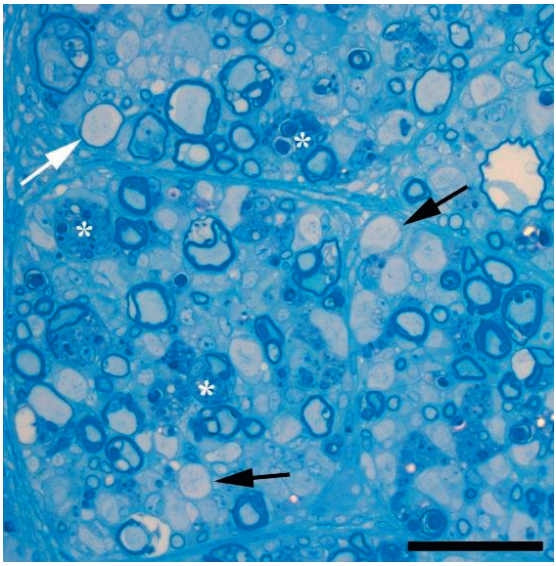

Figure 24. DRG/spinal nerve seven days after exposure to displacement and allogeneic NP. Note advanced loss of myelinated fibers, axon with thin myelin sheath (white arrow), denuded axons (black arrows), and aggregated debris (white asterisk). (Azure blue-methylene blue; Light microscopy; Bar:50µm)

There was significantly less nerve fiber damage in all the other experimental groups and no epineural granulation tissue was seen in these animals. Animals exposed to NP, notochordal cells in F12, chondrocyte-like cells in F12 or F12 medium alone displayed the same degree of nerve fiber damage as sham-operated animals, i.e., there were no significant differences between any of these experimental groups. No cellular inflammation could be detected in any of the samples (Fig. 25).

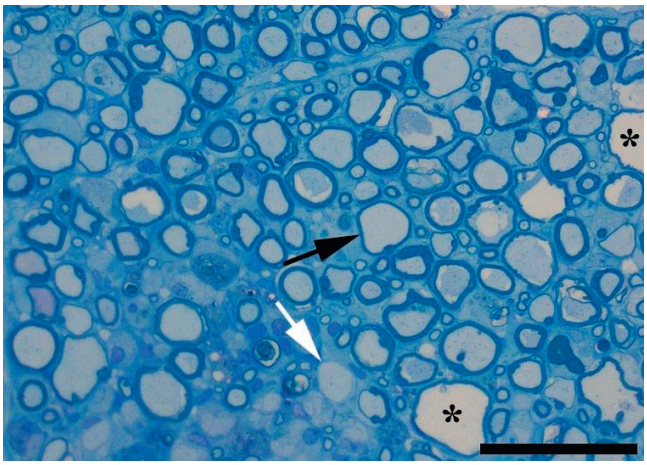

Figure 25. Spinal nerve seven days after exposure to notochordal cells. Note moderate loss of myelinated nerve fibers, nerve fiber with thin myelin sheath (black arrow), denuded axon (white arrow), fibers with thin myelin sheath and loss of axon (asterisk). (Azure blue-methylene blue; Bar:50 µm)

5.4.2 Morphology changes in Schwann cells

The number of myelinated nerve fibers with an enlarged outer Schwann cell compartment was significantly higher in animals exposed to NP, notochordal cells, chondrocyte-like cells and sham-operated animals, compared with naïve animals (Fig 26).

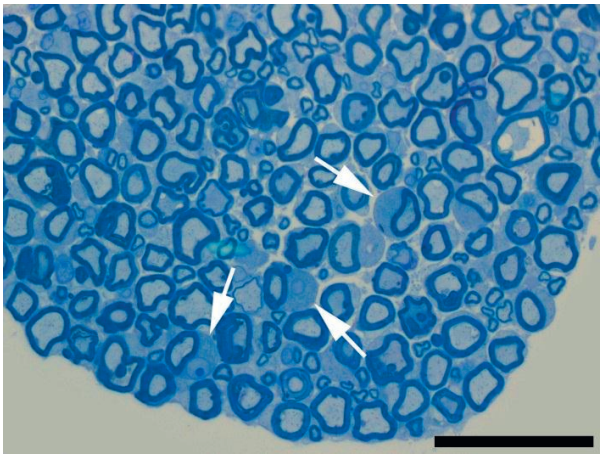

Figure 26. Myelinated nerve fibers with enlarged outer Schwann cell compartment (white arrows) seven days after exposure to notochordal cells. (Azure blue-methylene blue; Bar: 50 µm)

However, there were no differences between animals exposed to F12 medium and naïve animals with respect to the number of myelinated nerve fibers with an enlarged outer Schwann cell compartment. Compared with animals exposed to F12 medium, animals exposed to NP and sham-operated animals displayed a significantly larger number of Schwann cells with an enlarged outer compartment (Fig. 27).

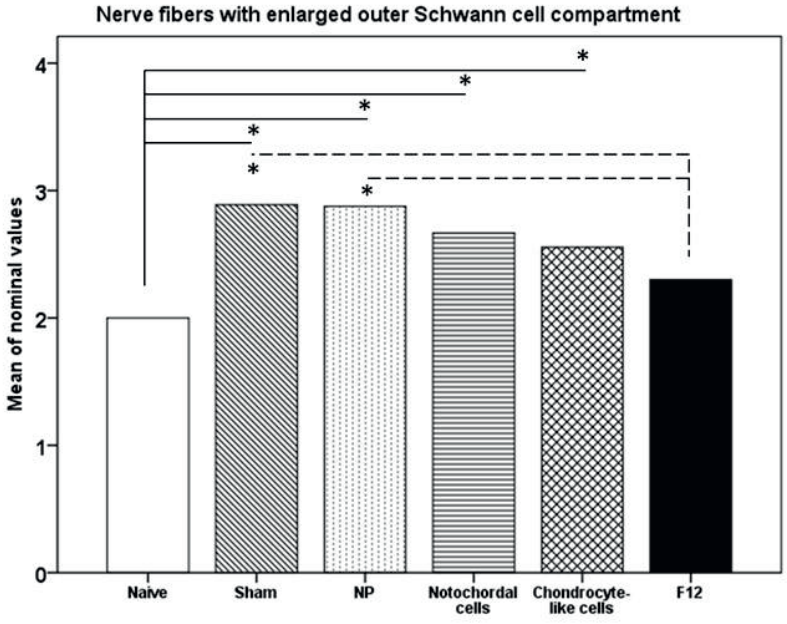

Figure 27. Mean nominal values for nerve fibers with an enlarged outer Schwann cell compartment. Compared with naïve animals, the number of myelinated nerve fibers with an enlarged outer Schwann cell compartment was significantly higher in sham-operated animals and animals exposed to NP, notochordal cells and chondrocyte-like cells (solid lines). Compared with animals exposed to F12 medium, the sham-operated animals and animals exposed to NP displayed a significantly larger number of myelinated nerve fibers with an enlarged outer Schwann cell compartment (dashed lines). From Study IV.

6 DISCUSSION

Intervertebral disc herniation is a common spinal disorder in which the herniated part of the disc causes mechanical compression of the nerve root. However, there is evidence indicating that the herniation-induced compression of the nerve root may not be the only cause of pain, e.g. as exemplified by findings that 35-70% of asymptomatic people without back and/or sciatic pain have disc herniation (Boden et al., 1990; Boos et al., 1995; Westrick et al., 2011). A disc herniation may be defined as a protrusion (the herniation still has contact with its origin), extruded (the herniation is larger than in the protrusion herniation and still has contact with its origin) or sequestered (a free disc fragment with no contiguous contact with the adjacent disc) (Westrick et al., 2011). Autologous nucleus pulposus from the intervertebral disc has been shown to affect nerve tissue *in vivo* as well as *in vitro* and effects of NP of this kind appear to be related to the cells of NP (Olmarker et al., 1993; Byröd et al., 1998; Kayama et al., 1998; Lidslot et al., 2000). The overall aim of this thesis was to investigate the effects of intervertebral disc cells, notochordal cells and chondrocyte-like cells, on neural tissue.

6.1 Notochordal- and chondrocyte-like cells

Previous studies have shown that NP can exert various effects on nervous tissue when applied locally *in vivo* (Olmarker et al., 1993; Olmarker et al., 1996; Byröd et al., 1998; Omarker and Myers, 1998; Brisby and Hammar, 2007; Yamashita et al., 2008; Sasaki et al., 2011). These effects seem to be mediated via the cells of NP, notochordal cells and chondrocyte-like cells, which affect the nervous tissue differently, as shown in the present thesis. However, one cannot exclude that there might also be involvement of matrix components of NP in the pathophysiology of the effects of NP *in vivo* on neural tissue.

The effects presented in this thesis include a reduction in the neurite outgrowth *in vitro,* when notochordal cells in intermediate and high concentrations, chondrocyte-like cells in high concentrations, and the combination of notochordal cells and chondrocyte-like cells in a concentration-dependent relationship were applied to nerve tissue culture explant (Study I). In Study II, the most interesting finding was the reduction in the neurite diameter of approximately 10%, in the presence of notochordal cells. It is known that in human situations modest variations in axon caliber

can occur, in various diseases (Griffin and Höke, 2005). Notochordal cells and chondrocyte-like cells affected the neuronal thalamic activity differently. Notochordal cells applied to the DRG resulted in a decrease in evoked thalamic activity. However, neither the application of intermediate or high concentration of chondrocyte-like cells, nor the application of a combination of the two cell types induced any changes in evoked thalamic activity (Study III). In Study IV, severe nerve fiber damage was seen in spinal nerves and DRG after nerve root displacement and the application of NP. In contrast, there was less nerve fiber damage in neural tissue exposed to NP, notochordal cells, chondrocyte-like cells or F12 medium compared with sham-operated animals. The number of myelinated nerve fibers with an enlarged outer Schwann cell compartment was higher in all experimental groups, compared with naïve animals, except for animals exposed to F12 medium. However, nerve fibers from animals exposed to NP and from sham-operated animals showed a larger number of Schwann cells with an enlarged outer compartment when compared with nerve fibers from animals exposed to F12 medium. Furthermore, a Schwann cell effect of this kind was not observed in animals following the application of notochordal cells or chondrocyte-like cells, suspended in F12 medium, when compared with F12 medium.

6.2 Number of disc cells and neural effects

The different effects on neural tissue demonstrated in this thesis may be related to the number of IVD cells and to the combination of IVD cell types applied to the nervous tissue. The large highly vacuolated notochordal cells appear to be the main cell type involved in the physiological effects incurred in nerve tissue as shown in this thesis. The notochordal cells in intermediate (Studies I,II and III) and high concentrations (Study I) had an effect on nerve tissue both *in vitro* and in acute *in vivo* models. In the disc herniation model used in Study IV, the notochordal cells and the chondrocyte-like cells did not affect the Schwann cells as compared to when NP was applied to the nerve root or in sham-operated animals.

In Study I, an increased number of chondrocyte-like cells (40,000 cells) induced a significant reduction in the neurite outgrowth. Ichimura et al described a morphologically heterogeneous population of small cells in rat NP and, in a study by Kim et al. the presence of notochordal cells with a chondrocytic phenotype was described in a rabbit model (Ichimura et al., 1991; Kim et al., 2009). They observed that notochordal cells morphologically differentiate into three distinct cell types; vacuolated cells, giant cells and polygonal cells. In terms of morphology and cytoplasmic area, chondrocyte-like cells are similar to polygonal cells and this may indicate

that an entirely pure population may not be obtained after cell sorting by size (Fig. 28). In the current situation, no specific markers of rat notochordal cells exist, so sorting by size was the best method available.

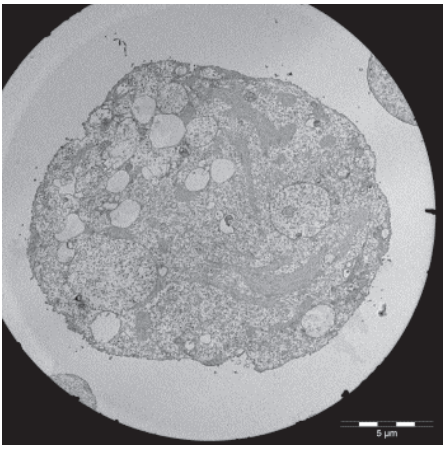

Figure 28. TEM image of a sorted chondrocyte-like cell from rat NP (Bar: 5µm)

As mentioned above, a large number of chondrocyte-like cells were required to obtain a negative effect on the outgrowing neurites in Study I (Fig.11). It is not possible to exclude effects on the neurite diameter, as analyzed in Study II, if a larger number of chondrocyte-like cells or a combination of the two cell populations had been applied to regenerating neurites *in vitro*. However, in Study III, neither a larger number of chondrocyte-like cells (Fig. 22) nor a combination of notochordal cells and chondrocyte-like cells (Fig. 23) resulted in any changes in evoked thalamic activity *in vivo* as compared to baseline. The lack of effects indicates that chondrocyte-like cells alone may not induce the increase in evoked thalamic activity, but the possibility that these cells may play a role in the complex pathophysiology of disc herniation and sciatica cannot be excluded.

6.3 Synergistic effects between disc cells

Within the *in vitro* model, used in Study I, it was possible to investigate the synergistic effect between the two cell populations on nerve tissue. The results indicated that the interaction response is different from the dose-response seen with only one cell type. Chondrocyte-like cells appear to have a “protective” effect on neurite outgrowth *in vitro*, as seen for example when

a high number of notochordal cells are applied in combination with chondrocyte-like cells, as compared to application of notochordal cells alone (Fig. 12). It has been demonstrated by other investigators that notochordal cells and chondrocyte-like cells may also have synergistic effects in other situations. These two cell types in combination resulted in increased proteoglycan production (Aguiar et al., 1999; Erwin et al., 2006). The two major glycosaminoglycans (GAGs) synthesized by disc cells are keratin sulphate GAG and chondroitin sulphate GAG (Maldonado and Oegema, 1992). It was shown by Castro and Kuffler that Schwann cell membrane-bound chondroitin sulphate proteoglycans inhibit neurite outgrowth (Castro and Kuffler, 2006). These results may support the assumption, that in the cell combination group in Study I, the inhibitory effect can depend on the increased production of chondroitin sulphate as a result of a synergistic effect between the two disc cells, in terms of increased proteoglycan synthesis. A combined effect of this kind was not investigated in Study II, but it would be of interest to investigate in future studies. The naturally occurring cell ratio in NP from rat spine discs corresponds to the number of cells which produced a significant inhibition of neurite outgrowth (1,500 notochordal cells and 28,500 chondrocyte-like cells) in Study I. This ratio was therefore used in Study III to investigate whether this cell ratio could influence the evoked thalamic activity (Fig. 23). A total of 30,000 cells was therefore applied to the DRG in the cell combination mentioned above, to evaluate whether the effect would resemble the increase in evoked neuronal responses following the acute application of NP previously observed *in vivo* (Brisby and Hammar, 2007). However, this cell combination did not induce any changes in evoked thalamic activity. The lack of effects may be due to the fact that the cell number and cell ratio applied *in vivo* were not optimal when it came to inducing thalamic responses such as those observed when NP was applied. An additional explanation may be that NP is a more complex tissue than a combination of two NP-derived cell populations. In Study III, the cells were applied to the DRG as the primary intervention, while Brisby and Hammar reported facilitating effects after the application of NP to the DRGs not only as a primary intervention but also to DRGs following prior exposure to adipose tissue. It has been demonstrated in a large number of *in vitro* studies that NP cells and other cell populations exert synergistic effects in term of positive stimulatory effects on matrix production (Le Visage et al., 2006; Niu et al., 2008; Vadala et al., 2008; Svanvik et al., 2009; Wei et al., 2009). The reported increase in evoked thalamic neuronal activity by NP might be a combined effect of NP and adipose tissue. For this reason, the study by Brisby and Hammar is not fully compatible with the lack of effects after the application of the combination of cells in Study III. Adipose tissue applied to the DRG has previously been used as a control group when evaluating

neuronal electrical activity, both in the spinal cord and in the adjacent nervous tissue, but with varying results. Some investigators have found similar effects from the application of NP and the application of adipose tissue (Anzai et al., 2002; Kallakuri et al., 2005), while others have demonstrated a difference between the two tissues (Takebayashi et al., 2001; Cuellar et al., 2004; Brisby and Hammar, 2007). Studying the effects of adipose tissue falls outside the scope of this thesis.

6.4 Experimental methods and techniques

Morphological and physiological analyses were used, alone or in combination, in the present thesis. Physiological effects were studied with the morphometric technique in Study I and with acute electrophysiological methodology in Study III. Using these physiological techniques, it was theoretically possible to distinguish differences in outcome as a result of effects from different disc cells on neural tissue. In Study I, a well-established computerized measurement technique was used to evaluate differences in neurite outgrowth for each DRG after the application of disc cells, notochordal cells and/or chondrocyte-like cells in seven different cell concentrations and in five different combinations of the two cell populations compared with the application of culture medium. A number of methods can be used to assess neurite outgrowth. Some studies have used dissociated DRG neuronal cultures (Wong et al., 2008) and other investigators have used explant cultures (Roufa et al., 1983; Lindwall and Kanje, 2005). It is possible to perform measurements of neurite outgrowth on images from live cultures or from cultures fixed and stained with the computerized measurement technique or with an ocular measurement procedure (Lidslot et al., 2000; Larsson et al., 2005). These two techniques live or fixed cells, are frequently used to measure neurite outgrowth. The technique used in this thesis is based on previous studies from our research group (Larsson et al., 2005), where a computerized quantitative assessment was used on living cultures. A quantitative assessment is often time-consuming and imposes rigorous demands on the observer. In Study I, the determination of neurite outgrowth was performed using an inverted microscope. The analysis was based on measurements of maximum outgrowth for each ganglion by measuring the neurite outgrowth in four directions, 90° from each other after 24 hours-time point I and after 48 hours-time point II (see 4.10.1 Study I). This procedure was performed by a single person blinded to the treatment. In Study II, a morphometric technique was used to measure the neurite diameter in cultures of DRG and neurites exposed to notochordal cells, chondrocyte-like cells or culture medium. In this study, we used fixated and ultra-thin sectioned

regenerated neurites and the examination was performed using a transmission electron microscope. The images were recorded and the neurite diameter was assessed at a later time by one observer. An acute electrophysiological technique, previously reported by Brisby and Hammar (Brisby and Hammar, 2007), was used in Study III. In Study III, it was demonstrated that the two cell populations, notochordal cells and/or chondrocyte-like cells affect the evoked thalamic activity differently, when applied to the DRG. It is very clear that the disc cells display a rapid physiological response which has been demonstrated in this thesis using various methods.

The small differences between the groups as seen with light microscopy (Study IV) and the lack of visible differences with electron microscopy analyses (Study II) between nerve tissues exposed to notochordal cells, chondrocyte-like cells or medium were surprising. However, the lack of detectable effects in the *in vitro* experiments (Study II) may be related to the limited number of cells and the ratio of cells. One explanation may be that the numbers of cells are insufficient or a combination of the two cell populations in an exact ratio is a prerequisite for obtaining an interactive response between the two cell types, resulting in neural changes. The exposure time of nervous tissue to the different cell types could be another important and essential factor which could influence the observed morphological changes. Study IV demonstrated significantly less nerve fiber damage and no epineural granulation tissue in myelinated nerve roots and DRG after the application of NP, notochordal cells, chondrocyte-like cells, F12 medium or in sham-operated animals compared with animals exposed to a combination of displacement of the nerve root/DRG and application of NP. However, the number of Schwann cells with an enlarged outer compartment was significantly higher in all the experimental groups compared with naïve animals, except for animals in the F12 group. It was not possible to distinguish the effects of the surgical trauma (sham animals) from the effects of the NP, notochordal cells or chondrocyte-like cells with respect to changes of this kind in Schwann cell morphology. A significantly larger number of nerve fibers with enlarged outer Schwann cell compartment were observed following exposure to NP and from sham-operated animals compared with nerve fibers exposed to F12 medium. Furthermore, no statistically significant difference was observed between nerve fibers with enlarged Schwann cells exposed to notochordal cells or chondrocyte-like cells compared with nerve fibers exposed to F12 medium. The notochordal cells and chondrocyte-like cells in Study IV were collected in F12 medium and this “protective ” effect of F12 medium may be related to the rinsing of soluble substances, such as interleukin-1β, (Il-1β), tumor necrosis factor (TNF) or connective tissue growth factor (CTGF) (Olmarker and Larsson, 1998; Aoki et al., 2002; Larsson et al., 2005; Erwin et al., 2006). F12 medium is tissue compatible

and is a useful carrier for different cells, i.e. cartilage cells in human cartilage transplantation surgery (Brittberg et al.,1994). It has been demonstrated that NP is able to produce several cytokines, including TNF-α, which may be of importance in the pathophysiology of disc herniation and sciatic pain (Takahashi et al., 1996; Olmarker and Larsson, 1998; Yoshida et al., 2005). In Study IV, there were no pronounced morphological changes, in contrast to previous studies in which NP or exogenous tumor necrosis factor (TNF-alpha) were applied to the DRG/nerve root *in vivo* (Omarker and Myers, 1998; Igarashi et al., 2000; Murata et al., 2004). The experimental set-up in the present study may explain the differences in morphological outcome. In previous models, a disc incision was made on the animals which were included in the histological investigation compared with the present experimental set-up in which NP was harvested from the tail discs of donor rats. The application of NP from donor rats might imply a shorter period of exposure, compared with animals with an incised disc with a prolonged leakage of NP.

6.5 Methodological strengths and limitations

6.5.1 *In vitro* model

The advantages of an *in vitro* model such as the DRG explant culture system used in this thesis, include the opportunity to determine whether a given cell population, cell combination or cell concentration exert direct action on neurite outgrowth. This DRG explant culture system is reproducible, it is well controlled and the analyses can be performed in a blinded manner. Separation of cells from NP provides opportunities to study and identify each cell population with respect to cell morphology, phenotype, RNA- and protein expression (Hunter et al., 2003; Hunter et al., 2003; Chen et al., 2006; Cao et al., 2007; Gilchrist et al., 2007), extracellular matrix production (Chelberg et al., 1995; Baer et al., 2001) and nutrient and other mechanical environments (Guilak et al., 1999; Bibby and Urban, 2004; Risbud et al., 2005; Hunter et al., 2007; Guehring et al., 2009). The viability of the cell populations was only affected to a minor degree by the cell-sorting procedure, see Study II. The viability of notochordal cells in all four studies was approximately 86% and among the chondrocyte-like cells the viability was approximately 97%. One advantage of the cell sorting procedure was that the gates determined for the reference population were used for all sorting experiments during the thesis. One of the limitations of using *in vitro* studies is the absence of systemic factors. Important limitations in Study II

were the high costs and the time-consuming process, which restricts the preparation of multiple samples for EM investigations.

6.5.2 *In vivo* models

The advantages of both acute and seven-day *in vivo* studies, as compared with the *in vitro* studies, was the presence of systemic factors and cells, such as cytokines, leucocytes and macrophages. The *in vivo* models can be expected to reflect the high complexity of the interaction involved between nervous tissue and nucleus pulposus and its components. One limitation in Study III was the use of anesthesia for all electrophysiological investigations, but it is not possible to perform the investigation in awake animals. The limitations in Study IV were that no electron microscopy investigation was performed and that the cell solution, consisting of notochordal cells or chondrocyte-like cells in F12 medium, applied to the DRG/nerve root may gradually diffuse from the site of application. One important limitation in the *in vivo* studies was the time-consuming experiments, with cell preparation in terms of cell separation and cell sorting just before the application of the cell populations in the experimental set-up. It would be desirable to freeze the notochordal cells and the chondrocyte-like cells for use at a later experimental time. However, this was not possible since the notochordal cells did not tolerate the freezing procedure well (personal observation).

6.6 Biomechanical and biological factors in disc herniation

The early observation by Mixter and Barr that lumbar intervertebral discs can herniate and cause mechanical nerve root compression, resulting in sciatic pain in the leg (Mixter and Barr, 1934), lead to a concept, which prevailed for several decades, that this was a mechanically induced condition. However, subsequent clinical studies were able to demonstrate that disc herniations are also common in individuals who have no back pain and no sciatic pain, so-called “silent disc herniations” (Hitzelberger and Witten, 1968; Boden et al., 1990; Boden et al., 1990). These clinical observations led to the formulation of a hypothesis that there might be components of nucleus pulposus that were able to induce nerve root inflammation through biochemical mechanisms, i.e. “chemical radiculitis” (Marshall and Trethewie, 1973; Marshall et al., 1977; Rydevik et al., 1984; McCarron et al., 1987). In 1993, it was demonstrated that the epidural application of autologous nucleus pulposus can produce structural and functional changes in porcine cauda equina nerve roots, without mechanical nerve root deformation (Olmarker et al., 1993). Neural changes of this kind induced by nucleus pulposus have subsequently been

confirmed by other investigators (Cavanaugh et al., 1997; Igarashi et al., 2000; Sasaki et al., 2011). It has also been shown that the application of nucleus pulposus to nerve roots, together with mechanical nerve root deformation, can cause pain-related behavioral changes (Omarker and Myers, 1998; Olmarker et al., 2003). Experimental studies have indicated that various cytokines, e.g. tumor necrosis factor, TNF, are involved in the pathogenesis of nerve root inflammation caused by the presence of nucleus pulposus near the neural tissue (Olmarker and Larsson, 1998; Igarashi et al., 2000). Further, experimental and clinical observations have shown that the systemic administration of various TNF inhibitors can limit or prevent nucleus pulposus-induced neural changes, including pain (Olmarker and Rydevik, 2001; Genevay et al., 2004; Onda et al., 2004; Murata et al., 2005; Cohen et al., 2009; Genevay et al., 2010). In the present thesis, the biological effects of nucleus pulposus on neural tissue have been further elucidated, by studies of the effects of intervertebral disc cells on neural tissue, thereby adding to the knowledge of the underlying mechanisms involved in these biological events. In Study IV, the experimental set-up included mechanical deformation of DRG/nerve roots, together with the local application of nucleus pulposus, resulting in pronounced neuropathological changes. These observations support previous findings that the combination of biomechanical factors (nerve deformation) and biological factors (effects of nucleus pulposus on neural tissue) are of importance in the pathophysiology of nerve root involvement in disc herniation and sciatica.

6.7 Clinical considerations

In general, the studies in this thesis support previous observations that the pathophysiology of disc herniation and sciatica is not only based on mechanical nerve root deformation, but also on a spectrum of biologic mechanisms. This thesis has clarified that some of these biologic mechanisms can be mediated via the cells in nucleus pulposus, i.e. notochordal cells and chondrocyte-like cells, and that these cell types have different effects on neural tissue. Such effects can be expected to be of pathophysiologic relevance in clinical situations like disc herniation and sciatica.

In patients with disc herniation, the clinical symptoms of sciatic pain are most likely based on a combination of the biologic effects of nucleus pulposus on the nerve root and mechanical factors leading to deformation of the neural tissue. Even in clinical situations where there are reasons to assume that the mechanical nerve tissue deformation is of comparable type and magnitude, there can be wide variations of symptoms between individuals, ranging from no or minimal sciatic pain to pronounced such

pain. One may speculate that there might be differences between individuals in terms of the cellular composition of nucleus pulposus and that such variation might contribute to the wide range of symptoms seen in patients with disc herniation. One may also speculate that further advancement regarding the understanding of the biology and pathophysiology of notochordal cells and chondrocyte-like cells may help to clarify the clinical variations of symptoms in patients with disc herniation and sciatica.

7 CONCLUSIONS

This thesis has evaluated the influence of disc cells on nervous tissue. The main conclusions, based on the experimental studies in the thesis, are as follows:

- Notochordal cells and chondrocyte-like cells from the rat NP both demonstrate a dose-dependent inhibitory effect on neurite outgrowth *in vitro*.
- Notochordal cells induce an average neurite diameter reduction of approximately 10% *in vitro*, compared with culture medium.
- The exposure of the DRG to notochordal cells and chondrocyte-like cells induce different effects on evoked thalamic activity. The application of notochordal cells alone results in a decrease in evoked thalamic activity.
- The neuropathology examinations reveal various morphology changes between the different experimental groups. The results indicate that complex mechanisms are involved in the interaction between the nucleus pulposus and spinal nerve tissue.

8 FUTURE PERSPECTIVES

The results of the studies in this thesis provide insight into the effects of the two NP-derived cell populations on nerve tissues. Clinically, patients with disc herniation may display different types and degrees of symptoms, despite similar mechanical influences by disc herniation on the nerve roots. One may speculate that the number of disc cells and the combination of disc cells in exact ratios may play an important role in the biologic effects of nucleus pulposus on nerve tissue, thereby providing a possible explanation for the variation in clinical symptoms between patients with disc herniation. Therefore, it would be of interest to investigate human disc cells in terms of cell types and cell ratios and attempt to correlate these aspects of intervertebral disc biology with clinical symptoms in sciatica.

Systemic factors such as cytokines in the serum may influence the interaction between disc cells and the nervous system, mechanisms which might be possible to analyze in both experimental and clinical studies.

It would also be of interest to continue the investigation of the reactions in nerve tissues after exposure to notochordal cells and chondrocyte-like cells with electron microscopy. Studies of this kind are in progress.

Moreover, it would be interesting to study the neural effects of two cell populations in a carrier, the properties of which should ensure that the cells are maintained at the site of application, in the same way as when the NP is applied to the nerve root.

SAMMANFATTNING

Introduktion: Ischias, d.v.s. smärta som strålar ut i benet, är ett vanligt kliniskt tillstånd som oftast orsakas av diskbråck i ländryggen. Nucleus pulposus (NP) i intervertebraldiskarna (IVD) kan vid diskbråck påverka närliggande nervrot inte enbart mekaniskt utan även genom biologiska mekanismer. Cellerna i diskvävnaden har i dessa sammanhang visats spela en viktig roll, men kunskapen avseende hur cellerna är involverade är ofullständig. Syftet med avhandlingsprojektet var att analysera interaktionen mellan cellpopulationer i IVD, notochordala celler respektive kondrocytliknande celler, och nervvävnad.

Material och metod: I delarbete I och II avlägsnades dorsala rotganglier (DRG) kirurgiskt från nyfödda Sprague-Dawley-råttor och dessa DRG placerades i odlingsskålar. Under 24 timmars odling växte neuriter ut från DRG och, därefter applicerades de två cellpopulationerna i olika koncentrationer, var och en för sig, eller i olika kombinationer. Efter ytterligare 24 timmars odling avslutades experimentet. I delarbete I mättes neuriternas utväxt digitalt efter 48, respektive 24 timmars odling och kvoten mellan de två mätningarna beräknades. I delarbete II preparerades DRG/neuriter för scanning- respektive transmissions-elektronmikroskopisk analys. Neuriters morfologi studerades och neuriters diameter beräknades. I delarbete III & IV studerades effekten av diskceller på nervvävnad i en akut respektive ”kronisk” diskbråcksmodell hos råtta. I den akuta modellen applicerades cellpopulationerna på L4 DRG och i den ”kroniska” djurmodellen på DRG/spinalnerv. Analyserna genomfördes med elektrofysiologisk respektive ljusmikroskopisk teknik.

Resultat: Notochordala- och kondrocytliknande celler hämmade neuritutväxt och minskade diametern hos neuriter *in vitro*, i ett dos-responsförhållande. De två cellpopulationerna påverkar på olika sätt den framkallade neurala aktiviteten i thalamus. En kombination av nervrotsförskjutning och applikation av NP på DRG/spinalnerv framkallade en omfattande nervskada. Mer diskreta förändringar, som nervfiber med förstorad Schwanncellcytoplasma, kunde observeras efter applicering av NP och/eller de olika cellpopulationerna.

Sammanfattning: Resultaten från denna avhandling visar att cellerna i NP, notochordala celler och kondrocytliknande celler, på olika sätt kan påverka nervvävnad. Fynden talar för att komplexa mekanismer är involverade i interaktionen mellan NP och nervvävnad.

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REFERENCES

Adams P, Muir H (1976). Qualitative changes with age of proteoglycans of human lumbar discs. Ann Rheum Dis 35: 289-296.

Adams P, Eyre DR, Muir H (1977). Biochemical aspects of development and ageing of human lumbar intervertebral discs. Rheumatol Rehabil 16: 22-29.

Agrawal A, Guttapalli A, Narayan S, Albert TJ, Shapiro IM, Risbud MV (2007). Normoxic stabilization of HIF-1alpha drives glycolytic metabolism and regulates aggrecan gene expression in nucleus pulposus cells of the rat intervertebral disk. Am J Physiol Cell Physiol 293: C621-631.

Aguiar DJ, Johnson SL, Oegema TR (1999). Notochordal cells interact with nucleus pulposus cells: regulation of proteoglycan synthesis. Exp Cell Res 246: 129-137.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2008). The Cell. New York, Garland Science, Taylor & Francis Group, LLC.

Anzai H, Hamba M, Onda A, Konno S, Kikuchi S (2002). Epidural application of nucleus pulposus enhances nociresponses of rat dorsal horn neurons. Spine 27: E50-55.

Aoki Y, Rydevik B, Kikuchi S, Olmarker K (2002). Local application of disc-related cytokines on spinal nerve roots. Spine 27: 1614-1617.

Argiro V, Johnson MI (1982). Patterns and kinetics of neurite extension from sympathetic neurons in culture are age dependent. J Neurosci 2: 503-512.

Argiro V, Bunge MB, Johnson MI (1984). Correlation between growth form and movement and their dependence on neuronal age. J Neurosci 4: 3051-3062.

Baer AE, Wang JY, Kraus VB, Setton LA (2001). Collagen gene expression and mechanical properties of intervertebral disc cell-alginate cultures. J Orthop Res 19: 2-10.

Bibby SR, Urban JP (2004). Effect of nutrient deprivation on the viability of intervertebral disc cells. Eur Spine J 13: 695-701.

Boden SD, Davis DO, Dina TS, Patronas NJ, Wiesel SW (1990). Abnormal magnetic-resonance scans of the lumbar spine in asymptomatic subjects. A prospective investigation. J Bone Joint Surg Am 72: 403-408.

Boden SD, McCowin PR, Davis DO, Dina TS, Mark AS, Wiesel S (1990). Abnormal magnetic-resonance scans of the cervical spine in asymptomatic subjects. A prospective investigation. J Bone Joint Surg Am 72: 1178-1184.

Bono C, Parke W, Garfin S (2011). Development of the Spine. Rothman-Simone The Spine. H. N. Herkowitz, Garfin, S.R., Eismont, F.J., Bell,G.R., Balderson, R.A. **1:** 2-14.

Boos N, Rieder R, Schade V, Spratt KF, Semmer N, Aebi M (1995). 1995 Volvo Award in clinical sciences. The diagnostic accuracy of magnetic resonance imaging, work perception, and psychosocial factors in identifying symptomatic disc herniations. Spine 20: 2613-2625.

Bray D, Bunge MB, Chapman K (1987). Geometry of isolated sensory neurons in culture. Effects of embryonic age and culture substratum. Exp Cell Res 168: 127-137.

Brisby H, Hammar I (2007). Thalamic activation in a disc herniation model. Spine 32: 2846-2852.

Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L (1994). Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 331: 889-895.

Byröd G, Rydevik B, Nordborg C, Olmarker K (1998). Early effects of nucleus pulposus application on spinal nerve root morphology and function. Eur Spine J 7: 445-449.

Byröd G, Otani K, Brisby H, Rydevik B, Olmarker K (2000). Methylprednisolone reduces the early vascular permeability increase in spinal nerve roots induced by epidural nucleus pulposus application. J Orthop Res 18: 983-987.

Cao L, Guilak F, Setton LA (2007). Three-dimensional morphology of the pericellular matrix of intervertebral disc cells in the rat. J Anat 211: 444-452.

Carlson B (2009). Human embryology and developmental biology.

Castro C, Kuffler DP (2006). Membrane-bound CSPG mediates growth cone outgrowth and substrate specificity by Schwann cell contact with the DRG neuron cell body and not via growth cone contact. Exp Neurol 200: 19-25.

Cavanaugh JM, Ozaktay AC, Yamashita T, Avramov A, Getchell TV, King AI (1997). Mechanisms of low back pain. A neurophysiologic and neuroanatomic study. Clinical Orthopaedics and Related Research: 166-180.

Chelberg MK, Banks GM, Geiger DF, Oegema TR, Jr. (1995). Identification of heterogeneous cell populations in normal human intervertebral disc. J Anat 186 (Pt 1): 43-53.

Chen J, Yan W, Setton LA (2006). Molecular phenotypes of notochordal cells purified from immature nucleus pulposus. Eur Spine J 15 Suppl 3: S303-311.

Cohen SP, Bogduk N, Dragovich A, Buckenmaier CC, 3rd, Griffith S, Kurihara C, Raymond J, Richter PJ, Williams N, Yaksh TL (2009). Randomized, double-blind, placebo-controlled, dose-response, and preclinical safety study of transforaminal epidural etanercept for the treatment of sciatica. Anesthesiology 110: 1116-1126.

Craig AM, Banker G (1994). Neuronal polarity. Annu Rev Neurosci 17: 267-310.

Cuellar J, Montesano P, Carstens E (2004). Role of TNF-alpha in sensitization of nociceptive dorsal horn neurons induced by application of nucleus pulposus to L5 dorsal root ganglion in rats. Pain 110: 578-587.

Dyck P, Thomas P (2005). Peripheral Neuropathy, Elsvier Saunders.

Erwin WM, Ashman K, O'Donnel P, Inman RD (2006). Nucleus pulposus notochord cells secrete connective tissue growth factor and up-regulate proteoglycan expression by intervertebral disc chondrocytes. Arthritis Rheum 54: 3859-3867.

Eyre DR, Muir H (1976). Types I and II collagens in intervertebral disc. Interchanging radial distributions in annulus fibrosus. Biochem J 157: 267-270.

Eyre DR, Muir H (1977). Quantitative analysis of types I and II collagens in human intervertebral discs at various ages. Biochimica et biophysica acta 492: 29-42.

Freshney R (1994). Culture of animals cells, Wiley-Liss.

Genevay S, Stingelin S, Gabay C (2004). Efficacy of etanercept in the treatment of acute, severe sciatica: a pilot study. Ann Rheum Dis 63: 1120-1123.

Genevay S, Viatte S, Finckh A, Zufferey P, Balague F, Gabay C (2010). Adalimumab in severe and acute sciatica: a multicenter, randomized, double-blind, placebo-controlled trial. Arthritis Rheum 62: 2339-2346.

Gilchrist CL, Chen J, Richardson WJ, Loeser RF, Setton LA (2007). Functional integrin subunits regulating cell-matrix interactions in the intervertebral disc. J Orthop Res 25: 829-840.

Gilson A, Dreger M, Urban JP (2010). Differential expression level of cytokeratin 8 in cells of the bovine nucleus pulposus complicates the search for specific intervertebral disc cell markers. Arthritis Res Ther 12: R24.

Greene E (1955) "Anatomy of the rat." Transations of the American Philoshopical Society **27**, 6-7.

Griffin J, Höke A (2005). The control of axonal caliber. Peripheral Neuropathy. P. J. D. a. P.K.Thomas. Philadelphia, Elsevier Saunders. **1:** 433-446.

Guehring T, Wilde G, Sumner M, Grunhagen T, Karney GB, Tirlapur UK, Urban JP (2009). Notochordal intervertebral disc cells: sensitivity to nutrient deprivation. Arthritis Rheum 60: 1026-1034.

Guilak F, Ting-Beall HP, Baer AE, Trickey WR, Erickson GR, Setton LA (1999). Viscoelastic properties of intervertebral disc cells. Identification of two biomechanically distinct cell populations. Spine 24: 2475-2483.

Gähwiler BH (1988). Organic cultures of neural tissue. Trends Neuroscience Journal 11: 480-484.

Götz W, Kasper M, Fischer G, Herken R (1995). Intermediate filament typing of the human embryonic and fetal notochord. Cell Tissue Res 280: 455-462.

Harrison RG (1907). Observation on the living developing nerve fiber. The Anatomical Record.

He Y, Baas PW (2003). Growing and working with peripheral neurons. Methods in cell biology, Elsevier Sciences. **71:** 17-35.

Hitzelberger WE, Witten RA (1968). Abnormal Myelograms in Asymptomatic Patients. J. Neurosurg. 28: 204-206.

Holm S, Maroudas A, Urban JP, Selstam G, Nachemson A (1981). Nutrition of the intervertebral disc: solute transport and metabolism. Connect Tissue Res 8: 101-119.

Hunter CJ, Matyas JR, Duncan NA (2003). The three-dimensional architecture of the notochordal nucleus pulposus: novel observations on cell structures in the canine intervertebral disc. J Anat 202: 279-291.

Hunter CJ, Matyas JR, Duncan NA (2003). The notochordal cell in the nucleus pulposus: a review in the context of tissue engineering. Tissue Eng 9: 667-677.

Hunter CJ, Matyas JR, Duncan NA (2004). Cytomorphology of notochordal and chondrocytic cells from the nucleus pulposus: a species comparison. J Anat 205: 357-362.

Hunter CJ, Bianchi S, Cheng P, Muldrew K (2007). Osmoregulatory function of large vacuoles found in notochordal cells of the intervertebral disc running title: an osmoregulatory vacuole. Mol Cell Biomech 4: 227-237.

Ichimura K, Tsuji H, Matsui H, Makiyama N (1991). Cell culture of the intervertebral disc of rats: factors influencing culture, proteoglycan, collagen, and deoxyribonucleic acid synthesis. J Spinal Disord 4: 428-436.

Igarashi T, Kikuchi S, Shubayev V, Myers RR (2000). 2000 Volvo Award winner in basic science studies: Exogenous tumor necrosis factor-alpha mimics nucleus pulposus-induced neuropathology. Molecular, histologic, and behavioral comparisons in rats. Spine 25: 2975-2980.

Ishihara H, Urban JP (1999). Effects of low oxygen concentrations and metabolic inhibitors on proteoglycan and protein synthesis rates in the intervertebral disc. J Orthop Res 17: 829-835.

Jack J, Ed. (1978). Studies in Neurophysiology. Some methods for selective activation of muscle afferent fibres. Cambridge, University Press.

Johansson F, Carlberg P, Danielsen N, Montelius L, Kanje M (2006). Axonal outgrowth on nano-imprinted patterns. Biomaterials 27: 1251-1258.

Johnson WE, Roberts S (2003). Human intervertebral disc cell morphology and cytoskeletal composition: a preliminary study of regional variations in health and disease. J Anat 203: 605-612.

Kallakuri S, Takebayashi T, Ozaktay AC, Chen C, Yang S, Wooley PH, Cavanaugh JM (2005). The effects of epidural application of allografted nucleus pulposus in rats on cytokine expression, limb withdrawal and nerve root discharge. Eur Spine J 14: 956-964.

Kawakami M, Tamaki T, Weinstein JN, Hashizume H, Nishi H, Meller ST (1996). Pathomechanism of pain-related behavior produced by allografts of intervertebral disc in the rat. Spine 21: 2101-2107.

Kayama S, Konno S, Olmarker K, Yabuki S, Kikuchi S (1996). Incision of the anulus fibrosus induces nerve root morphologic, vascular, and functional changes. An experimental study. Spine 21: 2539-2543.

Kayama S, Olmarker K, Larsson K, Sjogren-Jansson E, Lindahl A, Rydevik B (1998). Cultured, autologous nucleus pulposus cells induce functional changes in spinal nerve roots. Spine 23: 2155-2158.

Kim JH, Deasy BM, Seo HY, Studer RK, Vo NV, Georgescu HI, Sowa GA, Kang JD (2009). Differentiation of intervertebral notochordal cells through live automated cell imaging system in vitro. Spine 34: 2486-2493.

Krismer M, van Tulder M (2007). Low back pain (non-specific). Best Practice & Research Clinical Rheumatology 21: 77-91.

Larsson K, Rydevik B, Olmarker K (2005). Disc related cytokines inhibit axonal outgrowth from dorsal root ganglion cells in vitro. Spine 30: 621-624.

Le Visage C, Kim SW, Tateno K, Sieber AN, Kostuik JP, Leong KW (2006). Interaction of human mesenchymal stem cells with disc cells: changes in extracellular matrix biosynthesis. Spine 31: 2036-2042.

Lidslot L, Olmarker K, Kayama S, Larsson K, Rydevik B (2000). Nucleus pulposus inhibits the axonal outgrowth of cultured dorsal root ganglion cells. Eur Spine J 9: 8-13.

Lindwall C, Kanje M (2005). The role of p-c-Jun in survival and outgrowth of developing sensory neurons. Neuroreport 16: 1655-1659.

Lindwall C, Kanje M (2005). The Janus role of c-Jun: cell death versus survival and regeneration of neonatal sympathetic and sensory neurons. Exp Neurol 196: 184-194.

Maldonado BA, Oegema TR, Jr. (1992). Initial characterization of the metabolism of intervertebral disc cells encapsulated in microspheres. J Orthop Res 10: 677-690.

Marshall LL, Trethewie ER (1973). Chemical irritation of nerve-root in disc prolapse. Lancet 2: 320.

Marshall LL, Trethewie ER, Curtain CC (1977). Chemical radiculitis. A clinical, physiological and immunological study. Clin Orthop Relat Res: 61-67.

McCarron RF, Wimpee MW, Hudkins PG, Laros GS (1987). The inflammatory effect of nucleus pulposus. A possible element in the pathogenesis of low-back pain. Spine 12: 760-764.

Minogue BM, Richardson SM, Zeef LA, Freemont AJ, Hoyland JA (2010). Transcriptional profiling of bovine intervertebral disc cells: implications for identification of normal and degenerate human intervertebral disc cell phenotypes. Arthritis Res Ther 12: R22.

Mixter WJ, Barr JS (1934). Rupture of the intervertebral disc with involvement of the spinal canal. The New England Journal of Medicine 211: 210-215.

Murata Y, Onda A, Rydevik B, Takahashi K, Olmarker K (2004). Selective inhibition of tumor necrosis factor-alpha prevents nucleus pulposus-induced histologic changes in the dorsal root ganglion. Spine 29: 2477-2484.

Murata Y, Olmarker K, Takahashi I, Takahashi K, Rydevik B (2005). Effects of selective tumor necrosis factor-alpha inhibition to pain-behavioral changes caused by nucleus pulposus-induced damage to the spinal nerve in rats. Neurosci Lett 382: 148-152.

Niu CC, Yuan LJ, Lin SS, Chen LH, Chen WJ (2008). Mesenchymal Stem Cell and Nucleus Pulposus Cell Coculture Modulates Cell Profile. Clin Orthop Relat Res.

Olmarker K, Rydevik B, Nordborg C (1993). Autologous nucleus pulposus induces neurophysiologic and histologic changes in porcine cauda equina nerve roots. Spine 18: 1425-1432.

Olmarker K, Nordborg C, Larsson K, Rydevik B (1996). Ultrastructural changes in spinal nerve roots induced by autologous nucleus pulposus. Spine 21: 411-414.

Olmarker K, Iwabuchi M, Larsson K, Rydevik B (1998). Walking analysis of rats subjected to experimental disc herniation. Eur Spine J 7: 394-399.

Olmarker K, Larsson K (1998). Tumor necrosis factor alpha and nucleus-pulposus-induced nerve root injury. Spine 23: 2538-2544.

Olmarker K, Rydevik B (2001). Selective inhibition of tumor necrosis factor-alpha prevents nucleus pulposus-induced thrombus formation, intraneural edema, and reduction of nerve conduction velocity: possible implications for future pharmacologic treatment strategies of sciatica. Spine 26: 863-869.

Olmarker K, Storkson R, Berge OG (2002). Pathogenesis of sciatic pain: a study of spontaneous behavior in rats exposed to experimental disc herniation. Spine 27: 1312-1317.

Olmarker K, Nutu M, Storkson R (2003). Changes in spontaneous behavior in rats exposed to experimental disc herniation are blocked by selective TNF-alpha inhibition. Spine 28: 1635-1641; discussion 1642.

Olmarker K, Rydevik B, Kikucki S, Myers R (2011). Sciatica and Nerve Root Pain in Disc Herniation and Spinal Stenosis: A Basic Science Review and Clinical Perspective. Rothman-Simeone The Spine. H.

N. Herkowitz, Garfin, S.R., Eismont, F.J., Bell,G.R., Balderson, R.A. **1:** 129-145.

Omarker K, Myers RR (1998). Pathogenesis of sciatic pain: role of herniated nucleus pulposus and deformation of spinal nerve root and dorsal root ganglion. Pain 78: 99-105.

Onda A, Murata Y, Rydevik B, Larsson K, Kikuchi S, Olmarker K (2004). Infliximab attenuates immunoreactivity of brain-derived neurotrophic factor in a rat model of herniated nucleus pulposus. Spine 29: 1857-1861.

Otoshi K, Kikuchi S, Konno S, Sekiguchi M (2010). The reactions of glial cells and endoneurial macrophages in the dorsal root ganglion and their contribution to pain-related behavior after application of nucleus pulposus onto the nerve root in rats. Spine 35: 264-271.

Peacock A (1952). Observations on the postnatal structure of the intervertebral disc in man. J Anat 86: 162-179.

Radio NM, Mundy WR (2008). Developmental neurotoxicity testing in vitro: models for assessing chemical effects on neurite outgrowth. Neurotoxicology 29: 361-376.

Rajpurohit R, Risbud MV, Ducheyne P, Vresilovic EJ, Shapiro IM (2002). Phenotypic characteristics of the nucleus pulposus: expression of hypoxia inducing factor-1, glucose transporter-1 and MMP-2. Cell Tissue Res 308: 401-407.

Risbud MV, Fertala J, Vresilovic EJ, Albert TJ, Shapiro IM (2005). Nucleus pulposus cells upregulate PI3K/Akt and MEK/ERK signaling pathways under hypoxic conditions and resist apoptosis induced by serum withdrawal. Spine 30: 882-889.

Risbud MV, Shapiro IM (2011). Notochordal cells in the adult intervertebral disc: new perspective on an old question. Crit Rev Eukaryot Gene Expr 21: 29-41.

Roberts S, Caterson B, Evans H, Eisenstein SM (1994). Proteoglycan components of the intervertebral disc and cartilage endplate: an immunolocalization study of animal and human tissues. Histochem J 26: 402-411.

Robinson G, Gray T (1992). Electron microscopy Theory and practice of histological techniques. J. D. B. a. A. Stevens, Churchill Livingstone.

Ross M, Pawlina W (2006). Histology, A text and atlas with correlated cell and molecular biology, Lippincott Williams & Wilkins.

Roufa DG, Johnson MI, Bunge MB (1983). Influence of ganglion age, nonneuronal cells and substratum on neurite outgrowth in culture. Dev Biol 99: 225-239.

Rufai A, Benjamin M, Ralphs JR (1995). The development of fibrocartilage in the rat intervertebral disc. Anat Embryol (Berl) 192: 53-62.

Rutges J, Creemers LB, Dhert W, Milz S, Sakai D, Mochida J, Alini M, Grad S (2010). Variations in gene and protein expression in human nucleus pulposus in comparison with annulus fibrosus and cartilage cells:

potential associations with aging and degeneration. Osteoarthritis Cartilage 18: 416-423.

Rydevik B, Brown MD, Lundborg G (1984). Pathoanatomy and pathophysiology of nerve root compression. Spine 9: 7-15.

Sasaki N, Sekiguchi M, Shishido H, Kikuchi S, Yabuki S, Konno S (2011). A comparison of pain-related behavior following local application of nucleus pulposus and/or mechanical compression on the dorsal root ganglion. Fukushima J Med Sci 57: 46-53.

Schaeffer WI (1990). Terminology associated with cell, tissue, and organ culture, molecular biology, and molecular genetics. Tissue Culture Association Terminology Committee. In Vitro Cell Dev Biol 26: 97-101.

Stevens A, Lowe J (2001). Human Histology.

Svanvik T, Barreto Henriksson H, Karlsson C, Hagman M, Lindahl A, Brisby H (2009). Human Disk Cells from Degenerated Disks and Mesenchymal Stem Cells in Co-Culture Result in Increased Matrix Production. Cells Tissues Organs.

Takahashi H, Suguro T, Okazima Y, Motegi M, Okada Y, Kakiuchi T (1996). Inflammatory cytokines in the herniated disc of the lumbar spine. Spine 21: 218-224.

Takahashi N, Yabuki S, Aoki Y, Kikuchi S (2003). Pathomechanisms of nerve root injury caused by disc herniation: an experimental study of mechanical compression and chemical irritation. Spine 28: 435-441.

Takebayashi T, Cavanaugh JM, Cuneyt Ozaktay A, Kallakuri S, Chen C (2001). Effect of nucleus pulposus on the neural activity of dorsal root ganglion. Spine 26: 940-945.

Trout JJ, Buckwalter JA, Moore KC (1982). Ultrastructure of the human intervertebral disc: II. Cells of the nucleus pulposus. Anat Rec 204: 307-314.

Trout JJ, Buckwalter JA, Moore KC, Landas SK (1982). Ultrastructure of the human intervertebral disc. I. Changes in notochordal cells with age. Tissue Cell 14: 359-369.

Urban J, Roberts S (2006). The Intervertebral Disc: Normal, Aging and Pathologic. Rothman-Simeone The Spine. H. N. Herkowitz, Garfin, S.R., Eismont, F.J., Bell,G.R., Balderson, R.A. **1**.

Vadala G, Sobajima S, Lee JY, Huard J, Denaro V, Kang JD, Gilbertson LG (2008). In vitro interaction between muscle-derived stem cells and nucleus pulposus cells. Spine J 8: 804-809.

Walmsley R (1953). The development and growth of the intervertebral disc. Edinb Med J 60: 341-364.

Wei A, Chung SA, Tao H, Brisby H, Lin Z, Shen B, Ma DD, Diwan AD (2009). Differentiation of rodent bone marrow mesenchymal stem cells into intervertebral disc-like cells following coculture with rat disc tissue. Tissue Eng Part A 15: 2581-2595.

Weiler C, Nerlich AG, Schaaf R, Bachmeier BE, Wuertz K, Boos N (2010). Immunohistochemical identification of notochordal markers in cells in the aging human lumbar intervertebral disc. Eur Spine J 19: 1761-1770.

Westrick E, Sowa G, Kang J (2011). The Intervertebral Disc: Normal, Aging and Pathologic. Rothman-Simone The Spine. H. N. Herkowitz, Garfin, S.R., Eismont, F.J., Bell,G.R., Balderson, R.A. **1:** 97-108.

Windebank AJ, Blexrud MD (1986). Characteristics of neurite outgrowth from rat spinal ganglia: effects of serum and segmental level. J Neuropathol Exp Neurol 45: 683-691.

Wong JW, Brastianos HC, Andersen RJ, O'Connor TP (2008). A high-throughput screen to identify novel compounds to promote neurite outgrowth. J Neurosci Methods 169: 34-42.

Yabuki S, Kikuchi S, Olmarker K, Myers RR (1998). Acute effects of nucleus pulposus on blood flow and endoneurial fluid pressure in rat dorsal root ganglia. Spine 23: 2517-2523.

Yamashita M, Ohtori S, Koshi T, Inoue G, Yamauchi K, Suzuki M, Takahashi K (2008). Tumor necrosis factor-alpha in the nucleus pulposus mediates radicular pain, but not increase of inflammatory peptide, associated with nerve damage in mice. Spine 33: 1836-1842.

Yoshida M, Nakamura T, Sei A, Kikuchi T, Takagi K, Matsukawa A (2005). Intervertebral disc cells produce tumor necrosis factor alpha, interleukin-1beta, and monocyte chemoattractant protein-1 immediately after herniation: an experimental study using a new hernia model. Spine 30: 55-61.