Clusterin and Megalin in The Spinal Cord

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Abstract

Nerve injury induces up-regulation of the chaperone protein clusterin in affected neurons and adjacent astrocytes but the functional significance of this response is unclear. We find that motor neuron survival is significantly greater in clusterin(+/+) compared to (-/-) mice. These results suggest that endogenous expression of clusterin is neuroprotective after nerve injury. However, motor neuron survival in clusterin overexpressing mice was not different from that in wildtype mice. In contrast, treatment of neuronal cultures with clusterin-TAT recombinant protein is neuroprotective, including a positive effect on neuronal network complexity.

Since extracellular clusterin complexes are endocytosed after binding to various receptors, we examined the expression of known clusterin binding receptors in the spinal cord. We find that megalin is expressed in the nuclei of two cell populations in the mouse spinal cord: i) oligodendrocytes in late postnatal and adult spinal cord white matter, and ii) transiently (E11-15) in a population of immature astrocytes in the dorsal spinal cord. We find no correlation between clusterin and megalin in the intact or injured spinal cord. However, intranuclear localization of megalin, suggesting signalling properties, is supported by the co-localization with γ-secretase, the enzyme responsible for endodomain cleavage of megalin. Megalin deficient mice display a pronounced deformation of the dorsal part of spinal cord, an almost complete absence of oligodendroglial progenitor cells, and a marked reduction in the population of mature astrocytes at later prenatal developmental stages.

Taken together, our findings indicate that megalin is a novel signalling molecule for distinct populations of glial cells in the pre- and postnatal spinal cord. The functional role(s) of megalin is unknown. However, its expression patterns and cellular localization suggest that megalin regulates differentiation of oligodendrocytes and astrocytes in the prenatal spinal cord, as well as the function of myelinating oligodendrocytes in the postnatal spinal cord.

Keywords: nerve degeneration, hypoglossal nerve, chaperone, apolipoprotein, development, transcription factor, astrocyte, glial differentiation, myelin, cell signalling

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The stars have passed a judgement on me:
- Endless is error and misery.
- You who build towers that reach past the skies
  shall wander like beast, awaiting demise.
- All land shall crumble from under your feet
  till you shall perish of cold by the heat

To them the royal spirit replied:
stars locked in motion of self-rule denied!
Error is nothing but my freedom's toll
unventured land fills the depths of my soul.
Mournful is rustle of burial trees
Yet song of my life flows on the breeze.
I who build cities that reach past the skies
watch the stars fading with jealous cries.

“ANANKE” – Tadeusz Micinski
LIST OF PAPERS

The present Thesis is based on the following papers which will be referred to by their Roman numerals:

Paper I  - Wicher G, Aldskogius H. - Adult motor neurons show increased susceptibility to axotomy-induced death in mice lacking clusterin. 
_Eur J Neurosci. 2005 Apr;21(7):2024-8._

Paper II - Wicher G, Svenningsen AF, Charnay Y, Aldskogius H. - Effects on neurons of intracellular and extracellular clusterin – a study on clusterin over-expressing mice and _in vitro_. 
_Manuscript._

_J Neurosci Res. 2006 Apr;83(5):864-73._

_J Comp Neurol. 2005 Nov 14;492(2):123-31._

_Manuscript._

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ABBREVIATIONS

APC  Antigen presenting cell
ApoJ  Apolipoprotein J
APP  Amyloid precursor protein
ATP  Adenosine tri-phosphate
BLBP  Brain lipid-binding protein
BMP  Bone morphogenetic protein
BSA  Bovine serum albumin
CS  Chondroitin sulphate (e.g. CS56)
CNS  Central nervous system
CR3  Complement receptor type 3
DNA  Deoxyribonucleic acid
EAE  Experimental autoimmune encephalomyelitis
EphA4  Ephrin A4
FGFR  Fibroblast growth factor receptor
GSA-IB4  Griffonias implicifolia agglutinin - isolectin B4
GFAP  Glial fibrillary acidic protein
GRP  Glucose-regulated protein (e.g. Grp75, Grp78)
GST  Glutathione s-transferase
HO-1  Heme oxygenase 1
HSP  Heat shock protein (e.g. Hsp27, Hssp70, Hsp90)
HSPG  Heparan sulphate proteoglycan
IkB  Inhibitor of kappa B
IR  Immunoreactivity
LDL  Low-density lipoprotein
LDLR  Low-density lipoprotein receptor
LIF  Leukemia inhibitory factor
LRP  Low density lipoprotein receptor-related protein
MAG  Myelin-associated glycoprotein
NF-κB  Nuclear factor-kappa B
OMgp  Oligodendrocyte myelin glycoprotein
OPC  Oligodendrocyte precursor cell
ORP  Oxygen-regulated protein
PBS  Phosphate buffered saline solution
PCD  Programmed cell death
PCR  Polymerase chain reaction
pGK  Phosphoglycerate kinase
PNS  Peripheral nervous system
RBP  Retinol-binding protein
RNA  Ribonucleic acid
SCI  Spinal cord injury
Shh  Sonic hedgehog
SR-B1  Scavenger receptor B1
TBI  Traumatic brain injury
INTRODUCTION

1. Nervous system injury – a major medical problem

Injury to the nervous system includes peripheral nerve and root injuries, as well as direct brain and spinal cord injuries. These injuries often result in lifelong and disabling sensory, motor and cognitive deficits, and in the case of severe brain injury, often in death. In the age group between 15 and 24 years, traumatic brain injury (TBI) is a leading cause of mortality and, together with spinal cord injury (SCI), a leading cause of lifelong disability. Groups with very high risk of CNS damage also include elderly people and infants. The predominating reasons for the severe and longterm consequences of injury to CNS systems are the exceedingly fragile and sensitive neural tissue, the absence of spontaneous renewal of lost neurons, and the emergence of a non-permissive environment for axonal growth. Strategies to counteract injury induced nerve cell death and to promote regeneration of injured axons in the CNS are therefore prime goals for basic and applied research in neurotrauma. The mechanisms leading to nerve cell death and failure to repair damaged axons following neural trauma are likely to exist also in many other neurological disorders. Studies on experimental neurotrauma models are therefore relevant for our understanding of the pathogenesis of e.g. stroke and neurodegenerative disorders.

1.1. CNS consequences of injury are primary and secondary

Following direct injury to the brain or spinal cord neurons, glial cells and blood vessels are destroyed within an area, the size of which is dependent on the severity and type of injury. This primary injury is followed over the next hours to months of secondary degenerative events, which may be widespread and affect multiple regions in the CNS far away from the primary insult.

Components from dying cells, in combination with influx of exogenous cells and molecules create a harmful environment after CNS injury. Disturbances in the blood circulation lead to insufficient energy supply, hypoxia and hypoglycemia. As a result, the primary lesion becomes surrounded by a penumbra with a haemorrhagic front, which often continues to enlarge during the next hours. In this phase, cytotoxic factors, e.g. reactive species, from endogenous and exogenous cells as well as plasma start to interfere directly with nervous tissue homeostasis. Moreover, injury will induce an innate immune system response, often referred to as neuroinflammation, which may induce cell death directly (e.g. via apoptosis), or indirectly by promoting other secondary injury mechanisms.

Axons extending from damaged neurons within the primary injury zone may become demyelinated as a result of deranged physical and/or chemical homeostasis in their environment, or undergo complete disintegration (Wallerian degeneration). In both instances impulse
propagation fails with ensuing loss of function. Axons from nerve cell bodies situated outside the primary injury zone or penumbra may have been injured and undergo retrograde degeneration. Finally, neurons remote from the injury site may undergo trans-synaptic degeneration as a result of lost input (anterograde trans-synaptic degeneration) or loss of target neurons (retrograde trans-synaptic degeneration).

The CNS response to injuries progresses as an interplay between the injured neurons, endogenous non-neuronal cells, and, particularly in the case of direct CNS injuries, exogenous non-neuronal cells. The non-neuronal responses are complex and their overall consequences incompletely understood. On one hand, they appear to provide support for injured neurons. However, on the other hand, the activities of non-neuronal cells may contribute to nerve cell death, and are the basis for creating a non-permissive environment to neurite growth in the CNS.

2. Non-neuronal cells affected by neuronal injury

2.1. Endogenous (glial) cells – the most numerous cellular component of the CNS

First described by anatomist Rudolf Ludwig Karl Virchow (1821 – 1902) as “nerve glue”, glial cells are now divided into three main types: astrocytes, oligodendrocytes and microglia (Virchow, 1846). For a long time, glial cells were believed to play a secondary role in the nervous system, mainly providing structural, metabolic, and trophic support for neurons. However, recent evidence indicates that glial cells have important functions for the proper development and function of nervous system as well as for its reactions to injury (Volterra and Meldolesi, 2005; Pellerin, 2005; Pekny and Nilsson, 2005).

2.1.1. Astrocytes – neuronal collaborators and obstacle for regeneration

In 1906, the heavy metal impregnation (gold chloride- mercury bichloride technique) used by Santiago Ramon y Cajal (1852-1934), led to the first discovery of glial cells in CNS, the astrocytes. These are star-shaped, ubiquitous cells, irregularly shaped and with many long processes, which envelope all cellular components throughout the CNS.

Astrocytes are derived from progenitors in the neuroectoderm in the ventricular zone. In the spinal cord, astrocytes are commonly considered to develop in its dorsal part, although recent data indicate that their origin is more widespread with most of them developing from radial glial cells (Barry and McDermott, 2005). BMP4, expressed in the dorsal part of the spinal cord, promotes differentiation of glial progenitors to astrocytes in contrast to the ventral part, which is rich in Shh, a promoter of motor neuron and oligodendrocyte development.

In the uninjured CNS, astrocytes provide many supportive activities essential for neuronal function, including homeostatic maintenance of the
extracellular ionic environment and pH. In the context of signal propagation between neurons, astrocytes should be viewed as integral, modulating elements of synapses, where they play a fundamental role in the formation and maintenance of synaptic contacts, as well as in neurotransmission (Slezak and Pfrieger, 2003; Volterra and Meldolesi, 2005). Strongly connected with synapses and neuronal membranes, astrocytes actively remove released neurotransmitters as well as neuronal metabolites. Astrocytes also participate in transport processes between the CNS tissue and blood vessels, and contribute to the formation of the blood-brain barrier.

Through their intimate contacts with neurons as well as through tight connection with endothelial cells of blood vesicles astrocytes become the first glial cells in CNS to react to injury. Astroglial activation is characterised by hypertrophy, changes in gene expression as well as a potential to migrate to the site of injury. The release of pro-inflammatory growth factors from astrocytes contributes to activation of microglia and migration of haematogenous cells to the site of injury. Due to their general role as regulators CNS homeostasis in the intact CNS, as well as regulators of immune responses after injury, astrocytes are often considered as beneficial elements in injured nervous tissue (Anderson et al., 2003). However, at the same time reactive astrocytes are the major component of the glial scar, and produce molecules, that inhibit axonal regeneration (Silver and Miller, 2004). The glial scar is composed of astrocytes and their intertwined processes between which is laid down an extracellular matrix (e.g. collagen IV), resulting in an efficient physical barrier for growing axons. In addition, molecules such as proteoglycans NG2 and CS56, as well as the glycoprotein tenascin provide axon repulsive properties to reactive astrocytes. By reducing glial scar formation, growth of damaged axons is significantly improved in the spinal cord (Goldshmit et al., 2004).

2.1.2. Oligodendrocytes – myelin-forming cells and victims following CNS injury

The name oligodendrocyte was introduced by Rio Hortega (1882-1945). Oligodendrocytes are mainly known as myelin-forming cells. They extend numerous processes, each of which contacts and repeatedly envelopes a stretch of axon with subsequent condensation of this multissipral myelin forming membrane. The number of processes that form myelin sheaths from a single oligodendrocyte varies with the area of the CNS and possibly species, from about 40 in the optic nerve of the rat to one in the heavily myelinated pathways in the spinal cord of the cat (Hildebrand et al., 1993).

During spinal cord development, progenitors of the oligodendrocyte lineage are generated from the ventral part of the neural tube (ventricular zone). Through the antagonistic activities of the patterning molecules Shh and members of BMPs, oligodendrocytes differentiate and invade the white matter. In addition to the ventral source, oligodendrocytes also develop from central and dorsal parts of the ventral neuroepithelium (Vallstedt et al., 2005;
Degeneration of oligodendrocytes occurs in autoimmune disorders, as well as following spinal cord injury. A delayed loss of oligodendrocytes also occurs in areas undergoing Wallerian degeneration (Warden et al., 2001). Oligodendrocytes express several proteins (e.g. Nogo-A, MAG, OMgp), which are implicated in the neurite outgrowth inhibiting properties of the mature CNS environment (Kottis et al., 2002; Wang et al., 2002; Oertle et al., 2003). However, this view has been challenged by the finding that injured axons are actually able to grow rapidly along myelinated fiber tracts, although without branching into neighboring tracts, (“guard rail” through repulsive guiding) (Schwab and Schnell, 1991). Moreover, recent in vivo evidence suggests that MAG, a myelin component previously regarded as a neurite outgrowth inhibitor, actually promotes neurite outgrowth (Domeniconi and Filbin, 2005).

2.1.3. Microglia – more than tissue-specific macrophages

Microglia represent the third major population of glial cells within the CNS. The first descriptions of microglia-like cells are found in the works of William Ford Robertson (1867-1923) and Pio del Rio Hortega (1882-1945) (Robertson, 1900, Rio Hortega, 1919). Named by a pioneer in brain surgery, Wilder Penfield (1891-1976) “microglia” has since become a key component of CNS immunity (Penfield, 1924). The origin of microglia is still the subject of debate, but numerous studies in the last few decades have generally supported the view that microglia are derived from mesodermal precursor cells (haematopoietic lineage). Microglia play a crucial role in CNS homeostasis and is highly sensitive to changes in the microenvironment of the CNS. During development and maturation, microglia assist in the clearance of cells, which have undergone programmed cell death. In the mature brain and under physiological conditions, microglia display a resting form with a ramified morphology and serve the role of resident immune cells involved in host defence.

Interactions between neurons and microglia play a fundamental role in CNS injury responses. Microglia are readily activated in conditions such as trauma, stroke, inflammatory disease, infection as well as after peripheral nerve injury. After changes in the microenvironment of the CNS, microglia undergo dramatic morphological changes from resting ramified cells to fully activated microglia (phagocyte form). Activation of microglia is caused by injured neurons, which change rapidly their gene expression and release factors able to activate surrounding microglia (Schwaiger et al., 1998; Raivich et al., 1996; Raivich, 2005). Moreover, the activation process involves changes in number, protein expressions (e.g. receptors) and production of growth factors, cytokines and complement (Dong et al., 2004; Aloisi, 2001). As tissue-specific macrophages, microglia phagocytose degenerating neuronal elements.
Specific components of the complement system are crucial for efficient removal of degenerating peripheral myelin (Bruck, 1997; Dailey et al., 1998). CR3 is the main surface receptor involved in myelin recognition and uptake by resident and invading phagocytes. However, the complement cascade and activation of microglia into fully competent phagocytes are absent in the CNS (Reichert and Rotshenker, 1996; Liu et al., 1998).

2.2. Exogenous cells – hematogenous immune cells in an immune privileged site

In response to trauma, infection or autoimmune attack, a cascade of inflammatory processes occurs in the CNS. The acute inflammatory reaction is based on the activation of endogenous cells, and invading hematogenous mononuclear and polymorphonuclear cells.

Circulating monocytes respond to inflammatory mediators, migrate to the affected site in the CNS and become activated to a macrophage state. Following traumatic injury, monocytes/macrophages that appear in perivascular spaces and in the CNS parenchyma, increase during the first few days, both at the lesion site and in the adjacent perilesional areas (Beschorner et al., 2002). Besides its main function as phagocyte, macrophages are key elements as antigen presenting cells.

Lymphocytes are also crucial for immune responses in the CNS. During the first hours after injury, numerous T lymphocytes migrate into the affected nervous tissue through the open blood-brain barrier or through endothelial cells. In addition, self- and pathogen-specific T lymphocytes have the potential to mediate immunopathogenesis, contribute to secondary CNS injury and develop an autoimmune response. Thus, traumatic injury of the CNS spontaneously induces a T cell-mediated autoimmune response (Kipnis et al., 2002; Hauben et al., 2001). In experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis, numerous CD4+ T lymphocytes infiltrate nervous tissue in the first stage of the disease (Fee et al., 2003).

Similar to T and B cells, polymorphonuclear leukocytes (neutrophils, eosinophils and basophils) attracted by cytokines/chemokines, become activated and infiltrate the affected nervous tissue. These cells are predominantly observed in infection, stroke and trauma and also seem to play an important role in autoimmune disease. Intense activation of polymorphonuclear cells is associated with the production of a variety of cytotoxic factors, which contribute to secondary injury. In addition to their strong phagocytic potential, neutrophils have important roles in the modulation of acute inflammatory reactions. Recent evidence supports an immunoregulatory role of neutrophils in e.g. suppressing the capacity for T cells to induce an autoimmune response in EAE (Zehntner et al., 2005).
3. Cell destructive processes

3.1. Neuron degeneration

Traumatic brain injury, stroke, spinal cord injury or peripheral nerve injury affecting cell body compartments of neuron leads to destructive events, commonly referred to as neurodegeneration. These events are likely to develop through a complex interplay with genetic factors, stress responses, excitotoxicity, protein aggregation, insufficient energy supply, and failure of intracellular transport systems. Recently, there has been a growing interest in the role played by glial cells in the pathogenesis of neuronal injury and in dysfunction of molecular signaling pathways. In addition, glial cells themselves may undergo cell death as a result of primary and secondary injury. Death of oligodendrocytes, the most functionally most specific glial cells, will lead to demyelination of intact axons (Matute and Perez-Cerda, 2005). Although lost oligodendrocytes may be replaced from the pool of OPCs, this is a long-term process, and requires the presence of an optimal cellular and molecular environment (Aldskogius, 2005). Due to their ability to reproduce and migrate, degenerating microglia and astrocytes may be relatively easier to replace. However, loss of astrocytes probably has a significant influence on the homeostatic conditions of the injured area.

Axon injury will result in failure of impulse propagation and Wallerian degeneration (Waller 1850), i.e. progressive complete disintegration of the axon. This process is the result of rapid breakdown of the axonal cytoskeleton via ion-sensitive proteases (e.g. calpain). The axon is fragmented and collapses together with the surrounding myelin, leading to the formation of myelin ovoids. Concomitantly, astrocytes become activated and microglia cells are recruited to phagocytose the debris of degenerating axons and myelin. However, due to the incomplete activation of microglia, phagocytosis of myelin ovoids is slow and occurs over months or even years (Buss et al., 2005). Interruption of the axon may trigger processes leading to retrograde degeneration of affected neurons.

Neurodegenerative disorders as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and other, are associated with degeneration of specific neuronal populations. In these diseases, neurodegeneration is tightly connected with accumulation of certain abnormal polypeptides or mutated proteins inside or outside cell body which may cause neuronal death after years or decades. However, it is still a subject of debate whether aggregated proteins play a key role in the pathogenesis of these disorders, whether they represent harmless bystanders, or whether they could be beneficial to the cell by sequestering potentially toxic abnormal proteins (Ardley et al., 2005).

To some extent, neurodegeneration is not limited to pathological conditions of the CNS. Small-, or even large-scale elimination of synaptic terminals and axonal branches occur e.g. during development when neuronal connections are reorganized. Well established examples are: synapse
elimination at the neuromuscular junctions or selective collateral elimination of layer 5 neurons from mammalian neocortex (Nakamura and O'Leary, 1989; McLaughlin et al., 2003; Hashimoto and Kano, 2005).

3.2. Cell death – prolonged and “silent” or rapid and “loud”

Cell death in the nervous tissue is an integral part in the embryonic development, and the regulation of tissue homeostasis in normal conditions and pathologies. The classification of cell death is based on the underlying molecular pathways in combination with specific morphological features. Cell death may take place by apoptosis, by non-apoptotic forms necrosis or an intermediate form displaying both apoptotic and necrotic morphological characteristics. The mode of cell death and its morphological features is dependent on the cell type, signaling pathway, stimuli as well as environment (Ziegler and Groscurth, 2004).

Apoptosis is a highly controlled cell death, which can be induced by variety of physical or chemical factors from primary and secondary injury (see review). It also represents a strategic response to a stimulus that may result in damaging effects in the future e.g. modification in genetic material. During nervous system development, as well as adult life selected cells are destined to die through apoptosis (so called PCD) after performing a particular function at a specific time (e.g. radial glia). Apoptosis related molecular changes include energy-dependent activation of cascade proteins (e.g. caspases), which are followed by morphological changes in nucleus and cytoplasm. The main nuclear hallmarks are chromatin condensation, margination and nuclear fragmentation. Concomitantly, cells lose contact with neighboring cells, shrink and form apoptotic bodies containing cellular organelles and nuclear fragments. Finally, apoptotic bodies marked by the specific membrane surface molecular signatures are phagocytosed by surrounding cells. Apoptosis occurs with minimal or no inflammation due to the preservation of the cell membranes of the dying cells and their rapid phagocytosis.

Necrosis implies cell death, which is not programmed, non-energy dependent and in most cases accidental. Generally, necrosis an apoptosis appears following similar stimuli, however necrosis is associated with higher intensity. Morphologically, necrosis is characterized by changes in cell membrane permeability, dissociation of ribosomes from endoplasmic reticulum and disintegration of the nucleus. Based on morphology and the involvement of lysosomes, necrosis may be divided in: autophagic and nonlysosomal disintegration. In contrast to apoptosis, necrosis is typically associated with inflammation.
4. Endogenous cell protective factors

4.1. Stress response – Heat shock proteins protect cells against “cold” death

The cell stress response was first described by Ferruccio Ritossa in 1964, who observed distinct changes in Drosophila salivary gland chromosome (chromosomal puffs) in response to transient exposure of the insect to elevated temperatures (Ritossa, 1964). The stress response is an evolutionary conservative cell protection mechanism set into motion by various harmful stimuli, physical (e.g. mechanical injury, heat shock), chemical (e.g. oxidative stress, toxic factor) and biological (e.g. inflammation). Exposing cell to negative stimuli (stressors) lead to damage of their key structures, including DNA, RNA or proteins. Moreover, unfolded and damaged cell molecular compartments form irreversible precipitates and induce in severe cases cell death.

The cell stress response is controlled at the molecular level via two distinct mechanisms. The first mechanism comprises the chaperoning system and synthesis of cell stress proteins or so-called heat shock proteins, which function as molecular chaperones to assist protein folding, translocation and refolding of proteins partially denatured by stressors. The second mechanism comprises the synthesis of proteases and hydrolytic components of the ubiquitin-dependent proteasome for degradation of damaged and short-lived proteins.

As a result of CNS injury and the cell stress response various HSPs are expressed in affected neurons as well as in glial cells. Especially, Hsp70, Hsp90, ORPs, glucose-regulated protein (Grp75, Grp78) as well as small HSPs such as ubiquitin or HO-1, seem to play important protective roles in CNS stress responses (Rajdev et al., 2000; Richmon et al., 1995; Richmon et al., 1998; Sharp, 1998).

In vitro and in vivo studies as well as studies on transgenic animals over-expressing major inducible heat shock protein, Hsp70, demonstrate the neuroprotective effect of this protein (Franklin et al., 2005). Up-regulation of Hsp70 prior to sciatic nerve crush rescues motoneurons from cell death (Kalmar et al., 2002). In addition, administration of Hsp70 after axotomy inhibits motor and sensory neuron degeneration in spinal cord (Tidwell et al., 2004). Besides Hsp70, Hsp27 seems to emerge as an important protective molecule against a variety of stressors. In a model of motor neuropathy caused by premature axonal loss, Hsp27 appears to be a potent neuroprotective factor (Latchman, 2005).

5. Main factors in focus

Numerous observations show an association between clusterin expression and different neurological disorders (e.g., Alzheimer’s disease or gliomas) as well as with conditions with exaggerated cell death. These observations suggest that clusterin may play a role in the injured CNS (Choi-
Miura and Oda, 1996; Mattsson et al., 1998). As a secretory lipoprotein, clusterin uptake and degradation may be mediated by one of low density lipoprotein receptors or related endocytic receptors (e.g LRP). A likely functional candidate is a member of the low density lipoprotein receptor protein family, named megalin (Kounnas et al., 1995).

5.1. Clusterin

5.1.1. Structure

Clusterin or apolipoprotein J (ApoJ, SGP-2, SP-40, TRPM-2) is an amphipathic glycoprotein (75-80 kDa) with a high degree of conservation (70-85%). The protein is encoded from single structural gene from chromosome 8 as a full-length mRNA of about 1.6kb. As a heterodimer it is built from two subunits alpha- and beta-chains (Fig. 1) with 5 disulfide bridges and contains a 22 amino acid secretory signal polypeptide. 30% of mass of the mature protein is N-linked carbohydrates.

![Figure 1. Clusterin molecule structure (Jones et al. 2001).](image)

5.1.2. Expression and function

Clusterin was described in 1983 as a secreted protein widely distributed in physiological fluids as well as in a variety of tissues, including the central nervous system (Blaschuk et al., 1983; May and Finch, 1992).
Table 1. Clusterin ligands and their distribution (Trougakos and Gonos, 2002).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Ligand distribution</th>
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<tr>
<td>Complement components</td>
<td>Extracellular</td>
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<tr>
<td>Apolipoprotein A-I</td>
<td>Extracellular</td>
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<tr>
<td>Immunoglobulins</td>
<td>Extracellular</td>
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<tr>
<td>Lipids</td>
<td>Extracellular/intracellular</td>
</tr>
<tr>
<td>Beta-Amyloid peptide</td>
<td>Extracellular</td>
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<tr>
<td>Paraoxonase</td>
<td>Extracellular</td>
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<tr>
<td>gp330/megalin receptor</td>
<td>Extracellular</td>
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<tr>
<td>An extracellular protein (SIC) of</td>
<td>Extracellular</td>
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<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>Extracellular</td>
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<tr>
<td>Cell surface of <em>Staphylococcus aureus</em></td>
<td>Extracellular</td>
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<tr>
<td>Heparin</td>
<td>Extracellular</td>
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<tr>
<td>Prion peptide</td>
<td>Extracellular</td>
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<tr>
<td>Glycoproteins H, J and K</td>
<td>Cytoplasm (secretory granules)</td>
</tr>
<tr>
<td>TGF-beta receptors</td>
<td>Intracellular (plasma membrane)</td>
</tr>
<tr>
<td>Glutathione-S-transferase</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>Ku70, Ku86</td>
<td>Nucleus/plasma membrane/cytoplasm</td>
</tr>
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Many studies indicate that clusterin have intracellular as well as extracellular functions. Clusterin appears to be able to regulate processes such as lipid transport, clearance of cellular debris and intracellular signal transduction (Bailey et al., 2002). Secreted clusterin can influence cell-cell and cell-substratum interactions, thereby playing a role in cell aggregation and cell adhesion (Sivamurthy et al., 2001). Clusterin has also been suggested to participate in programmed cell death and aging (Park et al., 2003). Recently, the potential of clusterin to act as an ATP-independent, extracellular chaperone has been brought to attention. Clusterin expression is induced or up-regulated under various stressful conditions, such as ischemia (Wiggins et al., 2003); hypoxia (Han et al., 2001); intensive light (Wong et al., 2001); very low, non-toxic doses of ionising radiation (Criswell et al., 2003) or ethanol treatment (Dumont et al., 2002). The mechanisms underlying the chaperone properties of clusterin appear to include its ability to bind to hydrophobic regions of partially unfolded, stressed proteins, thereby avoiding their aggregation in the extracellular space (Carver et al., 2003).

5.1.3 Clusterin in nervous tissue

Clusterin and its mRNA is expressed ubiquitously in astrocytes and regionally in neurons (Pasinetti et al., 1994) (Xu et al., 2000; Wiggins et al., 2003), and is up-regulated following neural injury (Bellander et al., 1996; Liu et al., 1998; Liu et al., 1999) and in a wide range of CNS disorders. Clusterin appears to be involved in sporadic amyotrophic lateral sclerosis (Grewal et al., 1999), amyloid deposition in Alzheimer’s disease (Kulnane and Lamb, 2001; Torres-Munoz et al., 2001; DeMattos et al., 2002), and can
influence the structure, deposition and toxicity of alpha-synuclein in “alpha-synucleinopathies” such as Parkinson’s disease or dementia with Lewy bodies (Sasaki et al., 2002). Accumulation of clusterin is found in dying neurons in an animal model of epilepsy (Dragunow et al., 1995), in toxin-induced motor neuron degeneration (Tornquist et al., 1997), and in traumatically injured central nerve fibers (Liu et al., 1998). The strong association of clusterin with diseases characterized by protein misfolding, aggregation and precipitation suggests that clusterin is unable to counteract large-scale neurodegenerative processes (Poon et al., 2002). Clusterin is upregulated in axotomized motor neurons and adjacent astrocytes of the adult rat (Svensson et al., 1995; Mattsson et al., 1998).

5.2. Megalin

5.2.1. Structure

Low density lipoprotein receptor-related protein (LRP)-2/megalin is a multifunctional protein involved in cellular signalling and receptor-mediated endocytosis (Hjalm et al., 1996; Christensen and Birn, 2002; Muller et al., 2003).

![Figure 2. Megalin molecule structure.](image)
Megalin is one of the largest cell surface glycoproteins present in vertebrates (550-600kD), and belongs to the superfamily of low-density lipoprotein receptors (LDLR) (Saito et al., 1994); (Hjalm et al., 1996). Members of this family are characterized by high structural conservation and are expressed in a variety of species from invertebrates to mammals. Megalin contains a short cytoplasmic tail with two FXNPXY sequences, a single transmembrane domain and a large N-terminal extracellular domain (Fig. 2) composed of four clusters of LDL receptor A class repeats (ligand binding-complement-type repeats) (Saito et al., 1994; Hjalm et al., 1996). Megalin can specifically interact with several adaptor and scaffold proteins through the cytoplasmic tail domain (Oleinikov et al., 2000; Larsson et al., 2003; Nagai et al., 2003; Petersen et al., 2003; Biemesderfer, 2006).

Table 2. List of megalin ligands (Moestrup and Verroust, 2001).

<table>
<thead>
<tr>
<th>Vitamin-carrier complexes</th>
<th>TC-B12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vitamin D–binding protein</td>
</tr>
<tr>
<td></td>
<td>Retinol-binding protein</td>
</tr>
<tr>
<td></td>
<td>Vitamin A</td>
</tr>
<tr>
<td>Lipid-binding proteins</td>
<td>Apo B</td>
</tr>
<tr>
<td></td>
<td>Apo E</td>
</tr>
<tr>
<td></td>
<td>Apo J/clusterin</td>
</tr>
<tr>
<td></td>
<td>Apo H/2-glycoprotein-I</td>
</tr>
<tr>
<td></td>
<td>Apo(a)</td>
</tr>
<tr>
<td>Hormone/hormone-binding</td>
<td>PTH</td>
</tr>
<tr>
<td>proteins</td>
<td>Transhyretin</td>
</tr>
<tr>
<td></td>
<td>Thyroglobulin</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
</tr>
<tr>
<td>Drugs</td>
<td>Aminoglycosides</td>
</tr>
<tr>
<td></td>
<td>Polymyxin B</td>
</tr>
<tr>
<td></td>
<td>Aprotinin</td>
</tr>
<tr>
<td>Toxins</td>
<td>Trichosantin</td>
</tr>
<tr>
<td>Enzymes and enzyme</td>
<td>PAI-1</td>
</tr>
<tr>
<td>inhibitors</td>
<td>PAI-1-urokinase</td>
</tr>
<tr>
<td></td>
<td>PAI-1-tPA</td>
</tr>
<tr>
<td></td>
<td>Pro-urokinase</td>
</tr>
<tr>
<td></td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td></td>
<td>alpha-Amylase</td>
</tr>
<tr>
<td>Other</td>
<td>Albumin, RAP, Ig light chains, Ca²⁺, C1q</td>
</tr>
<tr>
<td></td>
<td>Lactoferrin, beta-2-Microglobulin, EGF</td>
</tr>
<tr>
<td></td>
<td>Prolactin, Lysozyme, Cytochrome c</td>
</tr>
<tr>
<td></td>
<td>Beta-1-Microglobulin, PAP-1</td>
</tr>
<tr>
<td>Vitamin-carrier complexes</td>
<td>TC-B12</td>
</tr>
<tr>
<td></td>
<td>Vitamin D–binding protein</td>
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<tr>
<td></td>
<td>Retinol-binding protein</td>
</tr>
<tr>
<td></td>
<td>Vitamin A</td>
</tr>
</tbody>
</table>
5.2.2 Expression and function

Megalin is expressed at high levels in the epithelia of the yolk sac, renal proximal tubules and intestine where it mediates uptake of various lipoproteins and vitamin complexes (Zheng et al., 1994; Kounnas et al., 1994; Christensen and Birn, 2002; Birn, 2006; Andreassen, 2006; Lin and Scanlan, 2005). Megalin is also expressed in the choroid plexus epithelium and ependymal cells of the adult brain (Zheng et al., 1994; Kounnas et al., 1994; Willnow et al., 1996; Chun et al., 1999), where it is likely to participate in transport processes between the vascular system, cerebrospinal fluid and brain tissue. Prenatally, megalin is expressed in the neuroepithelium (Muller et al., 2003; Drake et al., 2004), and is required for normal forebrain development. Mice with homozygous deletions of the megalin gene generally die perinatally, and manifest severe malformations, including absence of normal hemispheres, corpus callosum and olfactory bulb (Willnow et al., 1996). The mechanisms of megalin function during development of the brain appear to involve interactions with bone morphogenetic protein (BMP) 4 and sonic hedgehog (Shh), which are both megalin ligands (Spoelgen et al., 2005; McCarthy and Argraves, 2003; Morales et al., 2006). In contrast to a distinct role in brain development for megalin, there is little information on its possible function in the developing spinal cord. Strong expression of megalin has previously been shown only in the early embryonic neuroepithelium (6 – 8.5 days post coitum; Drake et al., 2004).

6. Background summary

CNS injury induces expression of clusterin, however, it is not known whether this up-regulation serves a neuroprotective function or not. Here, we have investigated role of clusterin for survival of neurons after various types of injury by comparing the extent of motor neuron survival in clusterin-deficient and wild type mice. In addition, we have explored the temporal and spatial expression of megalin, a potential clusterin receptor, in injured and normal spinal cord. However, we were struck by the minimal knowledge about the possible physiological and pathophysiological role(s) of megalin in the spinal cord. We have therefore undertaken a study of megalin expression in the prenatal as well as postnatal mouse spinal cord.
AIMS OF THE STUDIES

The general purpose of the present studies has been to extend our knowledge about the role of neuron-glial and glial-glial interactions in the spinal cord after injury.

Specific aims:

- to study the role of clusterin in injured motor neurons (Paper I)
- to study the role of intracellular and extracellular clusterin on neurons and neuronal cultures (Paper II)
- to study the possible role of megalin as a receptor for clusterin in the CNS (Paper III)
- to study the cellular localization and expression pattern of megalin in the postnatal spinal cord (Paper III)
- to study the cellular localization and expression pattern of megalin in the prenatal spinal cord (Paper IV)
- to study the role of megalin deficiency on early glial and neuronal development in the spinal cord (Paper V)
MATERIALS AND METHODS

Animals and experimental procedures
The studies were approved by the regional ethics committee for research on animals and carried out in accordance with the policy of the Society for Neuroscience. The animals were deeply anesthetised prior to all experimental procedures. Mouse strains used include:
- clusterin knock-out and wild type mice (Paper I)
- clusterin over-expressing (pGK) and wild type mice (Paper II)
- normal mice (NMR1) (Papers III and IV)
- megalin knock-out and wild type mice (Paper V).

Adult mice were subjected to transection or avulsion of the right hypoglossal nerve (Papers I and II). The animals were reanesthetised two and four weeks later, and the lower brain stem processed for analysis. Spinal cord and brain from 2, 9, 16, and 23 days old and adult mice were removed and processed for immunohistochemistry and immunochemistry (Paper III). Primary neural cell cultures were prepared from spinal cords of E13-E14 mouse embryos (Papers II and III). Spinal cord and/or brain were removed from embryos at stages E11, E13, E15, E16, E18 and E20 and processed for immunohistochemistry (Papers IV and V).

Genotyping (Paper I)
A polymerase chain reaction-based method was used to determine the genotype of the mice. Genomic DNA was isolated from tails or ears biopsies using a simplified mammalian DNA isolation procedure (Laird et al., 1991).

Primary cultures (Papers II and III)
Primary spinal cord cell cultures were prepared from embryonic gestation day 13-14 (E13-E14) mouse embryos as described previously (Bunge et al., 1988); (Svenningsen et al., 2003).

Cell lines (Paper III)
LLC-PK1 and HEK-293 cells expressing recombinant megalin were used.

Purification of a megalin-enriched protein fraction (Papers III and IV)
Purification of a megalin-enriched protein fraction from mouse kidney or liver (liver used as a negative control) was carried out as described by (Kanalas and Makker, 1990) with some modifications.

Purification of TAT-Clusterin (Paper II)
The cargo protein TAT-clusterin (TAT-CLU) was expressed in methylotrophic yeast Pichia pastoris (X33 strain) using the PIC6 alpha
vector containing a blasticidin resistance gene for the selection (InVitrogen, Switzerland). The TAT-CLU insert covering the entire mouse clusterin sequence (access number NM_013492) was obtained by RT-PCR using synthetic primers containing encoding sequences respectively for EcoR1-TAT and Not1 at their 3’ and 5’ endings. The Pic6 alpha vector containing the insert in frame was propagated in E.coli (TOP10; InVitrogen). After purification the recombinant plasmid was linearized by Pme1 for a chemical integration into Pichia by homologous recombination at the AOX1 locus (Pichia EasyComp kit; InVitrogen). Following selection by blasticidin in YPD medium, twenty colonies were checked for their ability to secrete TAT-CLU in the culture media after induction by 2% methanol for 48h. The supernatants were submitted to 1) SDS-PAGE electrophoresis and Comassie blue staining, 2) Western-blot analyses using specific antibodies against clusterin (see Imhof et al., 2006). Yeast clones revealing a strong signal in western-blot analysis were propagated in small (Wheaton; Switzerland) or large fermenter (Dr H. Boze, INRA, France). The cargo protein secreted in culture supernatants was purified by affinity chromatography using NiNTA Agarose according to the manufacturer instructions (Qiagen, Switzerland). The purified protein was dialysed against 0.1 mM sodium phosphate buffer, pH7.4, quantified (BCA Assay Kit), analyzed by western blot, lyophilized and kept at -80°C until use.

**TAT-Clusterin treatment of primary cultures (Paper II)**

10 days old primary cultures were treated with TAT-Clusterin protein in a final concentration of 50, 200 or 800ug/ml of medium for three days. Control cultures were treated with PBS. After incubation with clusterin and prior to staining, cell cultures were washed with PBS for 10 min and fixed.

**Antibodies to megalin (Papers III and IV)**

Anti-megalin antiserum (MM6) was produced by immunizing rabbits with glutathione S-transferase (GST) fusion protein produced from pGEX-5X-2 incorporating residues 4576-4630 of human megalin (Larsson et al., 2003). In addition, we used a previously characterized monoclonal antibody (E11), raised against the extracellular domain of megalin (Juhlin et al., 1987), as well as a rabbit antiserum produced against the entire human megalin (611) (Willnow et al., 1996).

**Immunohistochemistry (Papers I, II, III, IV and V)**

After fixation, the relevant tissues were immediately dissected out, post-fixed in the same fixative and cryoprotected overnight. Cryostat sections were made and slides were processed for immunohistochemistry. The sections were thawed at room temperature until water condensations on the slides had disappeared. Sections were pre-incubated with blocking solution for one hour at room temperature. After blocking, sections were
treated overnight with primary antibodies (Table 3) in the same solution used for blocking. After incubation with primary antibody, sections were rinsed in PBS, and the appropriate secondary antibodies were applied for four hours at room temperature. Sections were rinsed in PBS and mounted. The specificity of the MM6 antiserum was examined by preadsorption with antigen or with whole megalin. For other antibodies, the specificity of the immunoreactivity was verified by incubating adjacent sections without primary antibodies.

Table 3. Primary antibodies used in studies.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Species</th>
<th>Titer</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>beta-tubulin</td>
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<td>BioSite</td>
</tr>
<tr>
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<td>Clusterin</td>
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<td>Dr D. Holtzman</td>
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<tr>
<td>GFAP</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>1:100</td>
<td>Zymed Laboratories</td>
</tr>
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<td>GSA-IB4</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Vector Laboratories</td>
</tr>
<tr>
<td>GST-Megalin (MM6)</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>1:2000</td>
<td>Dr M. Larsson Uppsala University</td>
</tr>
<tr>
<td>Presenilin 1</td>
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<td>Mouse</td>
<td>1:500</td>
<td>Chemicon</td>
</tr>
<tr>
<td>Nestin</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>1:500</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>Megalin (611)</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>1:2000</td>
<td>Gift from Dr J. Herz</td>
</tr>
<tr>
<td>Megalin (E11)</td>
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<td>Mouse</td>
<td>1:80</td>
<td>Gift from Dr G. Åkerström and Dr C. Juhlin</td>
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<td>PDGFR alpha (C-20)</td>
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<td>Santa Cruz</td>
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<tr>
<td>RIP</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>1:1000</td>
<td>Iowa State Hybridoma Bank (Dr S. Hockfield)</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>1:200</td>
<td>Dako</td>
</tr>
</tbody>
</table>

Quantitative analyses (Papers I and II)

I. Neuronal profile number in hypoglossal nucleus

Serial cryostat sections from the lower brainstem of clu(-/-) and clu(+/+)(Paper I) as well as clusterin overexpressing and wild type (Paper II) mice were stained with cresyl violet. Motor neuron nuclear profiles in the hypoglossal nucleus on both sides were counted in every fifth section under the light microscope. The total number of motor neurons was estimated and the ratio of operated/non-operated motor neuron numbers was used as an index of motor neuron survival/degeneration. Statistical comparison was made with Student’s t test and p values < 0.05 were considered significant.

II. Fractal analysis of neuronal network in vitro

Network complexity was analysed by estimation of fractal dimension performed on binary images of neuronal cultures using the box-counting method (Smith et al., 1989). Prior to analysis, the relevant cultures were coded to minimize examiner bias. After beta-tubulin labelling, cultures were screened with regard to their overall appearance. Cultures were
selected for analysis when there were a minimum of 30 neurospheres with developed networks in the well. Ten random images (1600x1200 pixels) were made from each well of the selected neuronal cultures under the same magnification and exposure conditions. The binary overlay of a neuronal network was created using a thresholding procedure with the same settings for all images. Finally, the binary image of the neuronal network was reduced to its one-pixel outline. The estimation of fractal dimensions with the box-counting method was made according to standard rules. This method computes the number of grid squares (boxes) required to entirely cover an object. In practice, it is performed by superimposing a grid of squares over an object, followed by counting the number of squares containing, in this case, beta-tubulin labelled profiles. The logarithm of N(r), where N is the number of square occupying profiles and r is the size of one square, versus the logarithm of 1/r, gives a line with a slope of regression (S), which corresponds to the box dimension (D) by D=1-S. All images were analyzed using the public domain Image J software (version 1.33o; http://rsb.info.nih.gov/ij). Statistical analysis of fractal dimension data of TAT-clusterin treated and non-treated cultures was made with Student’s t-test and p values < 0.05 were considered significant.

Lysis of cells and tissue (Papers III and IV)

Lysates from spinal cord white matter (white matter was carefully separated from the gray matter), whole spinal cord or whole brain, were used for immunoblot experiments.

Lysates from LLC-PK1 cells or a human kidney cell line (HEK-293) expressing recombinant megalin were used for antibody blocking experiments (recombinant megalin expressing HEK-293) or immunoblotting experiments (LLC-PK1).

Immunoblot experiments (Paper III)

I. Lysates of mouse spinal cord, brain, a megalin-enriched kidney fraction and a megalin-free liver fraction were used for separation by Western Blotting.

II. Concentrated medium from serum-starved LLC-PK1 cells, lysates from LLC-PK1 cells, spinal cord white matter, a nuclear or a cytosolic fraction of LLC-PK1 cells were also used for separation by Western Blotting.

Microscopic analysis (Papers I, II, III, IV and V)

The slides were routinely viewed and analyzed in a fluorescence and light microscope. To resolve the intracellular localization of megalin immunoreactivity, selected sections were also examined in a confocal microscope.
RESULTS AND DISCUSSION

Clusterin – a neuroprotectant for axotomized motor neurons (Paper I)

Our findings confirm results from previous studies showing that clusterin is up-regulated in injured neurons (Svensson et al., 1995; Liu et al., 1998; Tao and Aldskogius, 1999; Calero et al., 2000; Van Beek et al., 2000; Klimaschewski et al., 2001). Our data also show that clusterin up-regulation is significantly greater after axon injury with rapid and virtually complete motor neuron death (avulsion) compared to injuries leading to only partial motor neuron loss (transection).

Figure 3. Quantitative analysis of motor neuron profiles in the operated (OP) and unoperated (UN) hypoglossal nucleus of clu(-/-) and clu(+/+) mice, two weeks after hypoglossal nerve avulsion (A) and four weeks after hypoglossal nerve transection (B). (Means ±SD, n=5 in each group, p<0.001).

The quantitative data from clu(-/-) and clu(+/-) mice show that constitutive expression of clusterin increases the survival potential of axotomized motor neurons. There was no significant motor neuron loss in clu(+/-) mice four weeks after hypoglossal nerve transection compared to a loss of almost 50% in clu(-/-) mice (Fig. 3). Motor neuron loss after nerve avulsion was of the same order of magnitude four weeks after injury in clu(+/-) and clu(-/-) mice. However, at two weeks after injury, clu(+/-) mice showed twice as many surviving
neurons compared to clu(-/-) mice (Fig. 3). Thus, in the absence of clusterin axotomized motor neurons i) are susceptible to death even after nerve transection, and ii) undergo death significantly faster after nerve avulsion. These findings clearly suggest that the pronounced clusterin up-regulation following avulsion injury reflects an attempt to support an intrinsic survival promoting response to the cell stress imposed by this lesion.

Numerous studies on cells and tissues outside the CNS have demonstrated that clusterin confers significant protection to a variety of cellular insults, such as leukocyte-induced lung injury, gentamicin-mediated renal epithelial cell cytotoxicity, and streptozotocin-induced degeneration of pancreatic islet cells (Park et al., 1999; Girton et al., 2002; Heller et al., 2003). In the nervous system, clusterin has been shown to inhibit the slow precipitation and aggregation of proteins, which can cause neuronal injury and serious neurodegenerative disorders like Alzheimer’s or Parkinson’s disease (Poon et al., 2002). Clusterin reduces the cytotoxicity of amyloid-beta (1-40) in primary cultures of rat mixed hippocampus (Boggs et al., 1996). Over-expression of clusterin in a mouse model of permanent focal cerebral ischemia, led to a significant reduction in the number of degenerating neurons (Wehrli et al., 2001). Administration of neuroprotective agents to neuroblastoma cells exposed to oxidant injury in vitro induced up-regulation of clusterin gene expression, suggesting that this protein counteracts neurodegeneration in this injury model (Sarang et al., 2002).

Results from previous studies indicate that retrograde degeneration induced by nerve or root avulsion produces extensive motor neuron death in rodents (see e.g. Hu et al., 2002; Ikeda et al., 2003). Moreover, degenerating motor neurons appear to die in part by apoptosis, which is associated with the formation of reactive oxygen species and oxidative damage to nucleic acids and proteins (Martin et al., 1999). Studies in vitro have demonstrated that oxidative stress induces up-regulation of clusterin gene expression and mediates resistance to apoptotic cell death, suggesting that clusterin is a stress protein. This view is reinforced by fact that the clusterin gene contains AP1 motifs, which are activated in the stress cascade, similar to heat shock protein family members. Furthermore, by its strong association with lipids and lipoproteins, clusterin can actively protect cells from apoptosis induced by extensive lipid peroxidation (Viard et al., 1999).

Clusterin is generally considered to be secreted and act as an extracellular chaperone (Carver et al., 2003). We find clusterin expression and up-regulation in astrocytes as well as motor neurons. However, we do not know whether clusterin is secreted from these cells in vivo. Previous studies suggest that astrocytes (Zwain et al., 1994; Morgan et al., 1995; Messmer-Joudrier et al., 1996) but not neurons (Pasinetti et al., 1994) are able to secrete clusterin. Thus, in the present situation, clusterin may act
within the axotomized motor neurons and/or in their extracellular environment. Clusterin may exert its extracellular activities e.g. by interaction with unfolded proteins or cell debris, and by neutralizing cytotoxic factors or immune complexes, which appear in the extracellular space after tissue injury. With its ability to bind to denatured proteins, clusterin can play role in protein recycling as well as in the protection and stabilization of the fragile microenvironment of the nervous tissue.

Clusterin may have functions in addition to its chaperone activities. Clusterin was shown to modulate cell-cell interactions, lipid transport, cell proliferation and immune responses. Recent data indicate that clusterin regulates IκB expression and NF-κB activity and indirectly influence death receptors (Santilli et al., 2003). These authors suggested, that the influence of clusterin on NF-κB activity could explain the opposing effects of clusterin in different experimental systems. On one hand, exogenously delivered clusterin can induce cortical neuron death in experimental hypoxia-ischemia model, while on the other hand, clusterin over-expression in human diploid fibroblasts can protect against cytotoxicity induced by ethanol or H₂O₂ (Han et al., 2001; Dumont et al., 2002). Thus, although clusterin possesses cell protective properties, the cellular localization, and time course and level of its up-regulation may actually promote cell death under certain conditions. However, our findings clearly indicate that clusterin is an endogenous survival-promoting molecule following axotomy.
Intracellular and extracellular clusterin - and different effect on neurons (Paper II)

In this study we used a similar mouse model of injury (nerve avulsion) with similar survival time (two weeks) as in our previous study on the role of clusterin in neuroprotection after axotomy (Wicher and Aldskogius, 2005). When comparing clusterin knock-out and wild type mice we found almost twice as many surviving neurons in wild type than in knock-out mice (Wicher and Aldskogius, 2005). However, our findings here show no significant improvement in motor neuron survival in clusterin over-expressing mice compared to wild type mice. These findings are in contrast to previous observations that clusterin over-expression promotes neuronal survival in a model of permanent focal cerebral ischemia (Wehrli et al., 2001). Over-expression of clusterin also protects human epidermoid cancer cells against heat shock and oxidative stress (Viard et al., 1999), as well as human diploid fibroblasts against cytotoxicity induced by ethanol or H₂O₂ (Han et al., 2001; Dumont et al., 2002).

**Figure 4.** Quantitative analysis of neuronal network complexity by estimation of fractal dimensions. Image preparation (left panel). Differences in fractal dimensions between treated and non-treated neuronal cultures (graph). (Means ±SD, * p<0.0001).

Since the cytoprotective effects of clusterin may be mediated through its extracellular actions, we examined the influence of extracellular clusterin on neuronal network complexity *in vitro*. We applied the method of fractal analysis, a field of applied mathematics based on fractal geometry and introduced by Mandelbrot (1982). This geometry has found numerous
applications as a means to quantitatively describe form and process complexity of natural objects. In general, fractal objects in space possess two important properties: self-similarity and scaling. In an ideal mathematical model, fractals are characterized by never-ending cascades with similar structural details. The self-similarity and scaling can be quantitatively estimated by the fractal dimension $D$. This measure describes the complexity of form and of the space-filling property of an object (Mandelbrot, 1982). Fractal analysis has emerged as an efficient method in neuroscience to describe e.g. complex neuronal morphology, including detailed neuronal branching characteristics (reviewed in (Fernandez and Jelinek, 2001), see also (Milosevic and Ristanovic, 2006). The method is therefore well suited to analyze network complexity in vitro.

Our data using fractal dimension analysis show significantly higher neuronal network complexity in TAT-clusterin treated cultures compared to controls (Fig. 4). These findings clearly suggest that extracellular clusterin is beneficial for neuronal process formation and extension. A previous study has shown that extracellular clusterin reduces the cytotoxicity of amyloid-beta (1-40) in primary cultures of rat mixed hippocampus (Boggs et al., 1996), supporting a survival promoting role of extracellular clusterin. As discussed above clusterin deficient mice show greater susceptibility to neuron degeneration, whereas over-expressing mice are generally more resistant than wild type mice in this respect. However, whether the effect of clusterin is mediated through its intra- and/or extracellular activities in these instances is not clear.

Clusterin is considered to be a secreted, extracellular chaperone (Carver et al., 2003). Secretion of clusterin by activated astrocytes (Messmer-Joudrier et al., 1996) may provide neuroprotection by interaction and/or neutralization of cytotoxic components (e.g. cell debris or unfolded proteins), which appears in the extracellular space after injury. With its ability to bind to denatured proteins, clusterin can play a role in protein recycling as well as in the protection and stabilization of the fragile microenvironment of the nervous tissue. On the other, exogenously delivered clusterin can exert cytotoxic effects, as shown by the induction of cortical neuron death in an experimental hypoxia-ischemia model (Han et al., 2001), and in the cell damaging effects of high concentrations of clusterin observed in the culture system used here.

Extracellular clusterin and its target molecules form complexes, which are subsequently bound to specific receptors and internalized for disintegration and elimination or for recycling of protein residues. Little is known about the receptors, which might be involved in internalization of clusterin complexes in the nervous system. Members of the LRP receptor family are likely candidates, but whether these are expressed in craniospinal somatic motor nuclei is unknown. We therefore undertook a systematic search for these receptors in the intact lower brainstem and spinal cord, as well as following various types of injury.
The lipoprotein receptor protein (LRP)2/megalin is selectively expressed in myelinating spinal cord white matter oligodendrocytes (Paper III)

In our search for possible clusterin binding receptors, we focused on lipoprotein receptor LRP-1, SR-B1, and LRP-2/megalin in the intact and injured spinal cord and brainstem. We found extensive expression of SR-B1 immunoreactivity (IR) in microglia, but no expression of LRP1 in the intact or injured spinal cord and brainstem (unpubl. observations). However, immunolabelling for megalin revealed an unexpected selective expression in the intact spinal cord white matter (Fig. 5), an expression that was unaffected by injury. This expression pattern differed profoundly from that in the brain, where megalin expression is restricted to ependymal cells and choroid plexus (Kounnas et al., 1994; confirmed by us). We therefore explored systematically the temporal and spatial expression pattern of megalin in the postnatal spinal cord (Fig. 5). Our main findings are that i) white matter oligodendrocytes in the mouse spinal cord, but not in brain, express megalin, and ii) that the cytoplasmic tail of this molecule is expressed in oligodendrocyte nuclei, suggesting that megalin is involved in membrane-nuclear signaling in these cells.

![Figure 5](image)

Figure 5. Megalin immunoreactivity in the mouse spinal cord at different postnatal stages. Strong immunoreactivity for megalin is present in ependymal cells of the central canal at all stages (A-D). No megalin-positive cells are present outside the ependymal layer at P2 (A). At P9, cells intensely immunoreactive for megalin are present in the ventral and lateral funiculus (B). At P16 (C) and P23 (D), the number of megalin-positive cells is increased and present throughout the white matter. Only single megalin-IR cells are found in the gray matter. Scale bars = 700 µm (A-B), 500 µm (C-D).

Megalin-IR cells in the spinal cord white matter were identified as oligodendrocytes based on their co-expression of transferrin, a marker for oligodendrocytes (Connor et al., 1993), as well as the absence of co-expression with the astroglial marker GFAP, and the microglial marker GSA-IB4. Megalin-IR cells were absent from the white matter of the brain, although labeling of ventricular ependyma and choroid plexus was consistent...
and distinct. Only occasional megalin expressing, transferrin-positive cells were found in the spinal cord gray matter. Thus, the white matter of the adult spinal cord is endowed with a substantial population of oligodendrocytes expressing megalin. The selective localization of megalin expression in spinal cord was also borne out by findings of megalin-IR oligodendrocytes in cultures from the spinal cord, but not cerebellum.

Megalin-IR cells in the spinal cord white matter were first observed at P9, and increased in number until P23, when the adult pattern was seen (Fig. 5). None of the megalin-IR cells expressed the PDGF receptor α or the transcription factor Olig2, markers for oligodendrocyte precursors, supporting the conclusion that megalin is required for functions of mature oligodendrocytes. The temporal and spatial pattern of megalin-IR oligodendrocytes indicates that megalin expression is linked to the formation and maintenance of myelin along axons of long spinal pathways. An intriguing issue in this case, is why megalin is not expressed by oligodendrocytes associated with the same pathways at higher levels of the CNS.

Morphological heterogeneity among oligodendrocytes reflects whether they participate in myelination of prospective large or small fibers. Oligodendrocytes, which myelinate large caliber axons in the spinal cord are typically associated only with one internode (Anderson et al., 1999). In contrast, oligodendrocytes myelinating the much thinner axons in the corpus callosum are responsible for many internodes. Oligodendrocytes in the chicken spinal cord, but not brain white matter, are labeled with antibody T4-O (antigen unidentified) (Anderson et al., 2000). Homozygous deletions of the Src tyrosine kinase Fyn results in severe myelin deficit in the forebrain, but not in the spinal cord (Sperber and McMorris, 2001). Thus, there are precedents of differential oligodendroglial phenotypes in the brain and spinal cord. However, it is still obscure why brain oligodendrocytes associated with pathways, which originate or terminate in the spinal cord, do not express megalin.

The majority of oligodendrocytes in the spinal cord originate from its ventral part under the influence of Shh (for review on sonic hedgehog see e.g. Miller, 2002). Smaller populations of oligodendrocytes arise from the dorsal spinal cord, mainly at later developmental stages and independent of Shh signaling (Cai et al., 2005; Fogarty et al., 2005; Vallstedt et al., 2005). The different sites of oligodendrocyte origin may reflect future spatial and functional differences, e.g. between oligodendrocytes in the spinal cord gray and white matter. Differences in the pathways regulating development and maturation of oligodendrocytes in the spinal cord and brain white matter may also result in spatially different cellular phenotypes.

Nuclear presence of megalin carboxyterminal fragment in oligodendrocyte nuclei in vivo as well as in vitro indicates that megalin does not play the traditional role of a receptor for cargo transport into the cytoplasm. Similar to e.g. the Notch receptor (Schroeter et al., 1998) and
APP (Lendon et al., 1997), megalin can undergo the process of regulated intramembrane proteolysis, thereby linking receptor activation and intracellular signaling (Petersen et al., 2003; Zou et al., 2004). The colocalization of presenilin-1 and megalin-IR indicate that the enzymatic requirements for gamma-secretase mediated endodomain cleavage of megalin are present in spinal cord white matter oligodendrocytes. By the cleavage and intranuclear translocation of the cytoplasmic tail, megalin may act as a transcription regulator. We suggest that the megalin distribution reflects functional requirements, which are unique to the heavily myelinated long distance ascending and descending pathways in the spinal cord white matter.

Megalin may play an important role in spinal cord white matter pathology (Besler and Comoglu, 2003). Deficient cholesterol transport due to megalin dysfunction might contribute to the pathogenesis of demyelinating and neurodegenerative disorders (Cutler et al., 2004). Deficiency of vitamin B12 is associated with demyelination and loss of function in long-ranging, ascending spinal systems, so-called subacute combined degeneration (Lovblad et al., 1997; Misra et al., 2003). Megalin can bind and endocytose the B12 complex, transcobalamine (Christensen and Willnow, 1999), and the efficiency of this uptake mechanism may therefore influence the pathogenetic process in this neurological disorder.

Megalin has been shown to bind clusterin in vitro. However, the absence of megalin expression in motor nuclei following injury, suggests that megalin is not involved in binding and uptake of clusterin complexes in this situation. The scavenger receptor B1 appears to be a likely candidate in this regard. Clusterin is expressed by astrocytes in gray as well as white matter, and markedly up-regulated along degenerating white matter pathways (Liu et al., 1998) and (Liu et al., 1999). Megalin may therefore be involved e.g. in the clearance of complexes formed between clusterin and abnormal proteins, which are deposited in abundance during Wallerian degeneration in the CNS.
Megalin is transiently expressed mainly in immature astrocytes in the prenatal spinal cord (Paper IV)

Our finding in Paper III strongly indicated that in contrast to the brain, megalin has a unique role in the formation and/or maintenance of myelinated pathways in the spinal cord. However, megalin plays a crucial role in brain development as evidenced by the severe brain malformations in megalin deficient mouse fetuses. This raises the question whether megalin has a role in the early spinal cord development as well. Previous studies have shown that megalin is expressed in the neural ectoderm, neural plate and neural tube during the early embryonic period (up to E9.5), but later prenatal stages appear not to have been studied. We therefore undertook a systematic investigation of megalin expression in the mouse spinal cord from E11–E18.

![Figure 6](image.png)

**Figure 6.** Megalin immunoreactivity(IR) in the mouse spinal cord at different prenatal stages. Strong immunoreactivity for megalin is present in ependymal cells of the central canal at all stages (A-D). Megalin-positive cells outside the ependymal layer are first found at E11 (A). Confocal image shows megalin-IR in cytoplasm of ependymal cells (A; insert). At E13, cells intensely immunoreactive for megalin are present in the dorsal spinal cord (B). At E15 (C) and E18 (D), the number of megalin-positive cells has decreased and is restricted to single cells in the gray matter close to the midline of spinal cord. Scale bar = 250 µm.

We find that megalin is expressed in a subpopulation of cells located in the dorsal part of the developing spinal cord from E11 to about E15 (Fig. 6), and that the vast majority of these cells also express vimentin.
Astrocytes are also commonly considered to develop in the dorsal part, although recent data indicate that their origin is more widespread with most of them developing from radial glial cells (Barry and McDermott, 2005). Both immature astrocytes and radial glia express vimentin. However, we did not find co-localization of vimentin with the radial glial marker BLBP, indicating that the vimentin+/megalin+ cells are immature astrocytes, which do not pass through a radial glial stage. If so, the megalin+/vimentin+ cells may develop into the population of white matter astrocytes, which develop directly from glial precursors to astrocytes (Barry and McDermott, 2005).

A small population of megalin-IR cells, presumptive dorsal horn neurons, was identified, as evidenced by the presence of cells co-expressing megalin and class III beta-tubulin, a marker for immature neurons. Finally, the small population of cells expressing megalin- and nestin-IR in the dorsal part of the developing spinal cord is likely to represent early developmental stages of either astrocytes or dorsal horn neurons. Oligodendrocytes develop from the ventral spinal cord during the time period when we find megalin expression (Noll and Miller, 1993), although at later stages oligodendrocytes also develop from the dorsal part (Vallstedt et al., 2005). Thus, oligodendrocytes appear to develop entirely without expressing megalin.

The development of cell populations and connections in the spinal cord is regulated by a tightly controlled sequence of transcription factors, growth factors, adhesion and guidance molecules, as well as their corresponding receptors. The expression of FGFR3 (Pringle et al., 2003) and the Notch effector gene Hes1 (Wu et al., 2003) coincide in space and time with our finding of megalin+/vimentin+ cells. Both FGFR3 and Hes1 appear to promote the development of glial progenitors to an astroglial fate. BMPs and retinoic acid are also present in the dorsal part of the developing spinal cord within the appropriate time frame, and are particularly relevant, since BMP and retinol-binding protein (RBP) carrying retinol, the precursor of retinoic acid, are ligands of megalin (McCarthy and Argraves, 2003; Spoelgen et al., 2005). BMP4 promotes differentiation of glial progenitors to astrocytes in the brain (Yanagisawa et al., 2001; Gomes et al., 2003; Liu et al., 2004), whereas retinoic acid is a major determinant of dorso-ventral and antero-posterior patterning of the spinal cord (Wilson et al., 2004). Furthermore, retinoic acid regulates the function of Hes family genes, which in turn control differentiation along the astroglial lineage (Wakabayashi et al., 2000; Wu et al., 2003).

Megalin has a well established role as an endocytotic, scavenger-like receptor in absorptive epithelia for low molecular weight proteins and vitamins. Recent findings have shown, however, that megalin can serve as a signaling molecule for events at the cell surface to the nucleus (Zou et al., 2004). Following ligand binding, the cytoplasmic tail of the megalin molecule is cleaved by gamma-secretase and transported to the nucleus, where it might influence gene transcription, either directly as a transcription
factor or indirectly by regulating the activity of other transcriptional processes. This function is particularly relevant in the context of our findings, since strong megalin-IR was consistently observed in the nuclei of labeled cells. Thus, binding of BMPs and/or carrier proteins such as RBP to megalin may not only be followed by internalization of the ligands, but also have a critical role in spatio-temporal patterning and cell fate determination in the spinal cord.

Binding of BMPs to megalin expressing cells may therefore direct their development into the astroglial lineage. Shh, which is produced in the floor plate, is also a ligand of megalin (McCarthy and Argraves, 2003). Shh plays a crucial role in promoting the development of motor neurons and oligodendrocytes in the ventral spinal cord, whereas astroglial differentiation is Shh independent. For reasons that are presently obscure, developing astrocytes may require the presence of megalin and scavenging of Shh prior to their final differentiation. Shh is a prime regulator of CNS development; stimulates oligodendroglial development, and at the same time restricts the ventral extension of astroglial precursors (Agius et al., 2004). Endocytosis of Shh by megalin thereby eliminates it from the microenvironment as a promoter of the oligodendroglial lineage, and permits astroglial development in the dorsal spinal cord. Furthermore, Shh expression is lost in the forebrain of mice with homozygous deletions of the megalin gene (Spoelgen et al., 2005), whereas BMP4 is increased above normal levels.
Critical changes in early glia development in the spinal cord of megalin deficient mice (*Paper V*)

Our findings show that absence of megalin is associated with severe malformation of the caudal spine as well as abnormal development of the spinal cord including the development of oligodendrocytes and astrocytes (Fig. 7). These findings indicate that megalin plays a critical role in the regulation of glial lineage development in the prenatal spinal cord. Since megalin(-/-) mice die before or shortly after birth (Willnow et al., 1996), the longterm consequences of megalin deficiency for spinal cord development and functions cannot be assessed in the animal model we have studied here.

**Figure 7.** Embryonic spinal cord of megalin deficient mice (stage E20). Cresyl violet staining shows marked malformation of the spine in megalin(-/-) mice. NG2 and Olig2 positive oligodendrocyte precursor cells are absent, and there is a marked reduction in the number of GFAP-positive astrocytes in megalin(-/-) mice.

The cell populations in the spinal cord develop in distinct spatial and temporal patterns. Generally, motor neurons and oligodendrocytes develop from the ventral part, whereas dorsal horn neurons and astrocytes develop from the dorsal part (Jessell, 2001). Previous studies have shown that cells expressing Olig2 and/or NG2 share characteristics with OPCs; however, NG2 expression is not restricted only to OPCs (Stallcup, 2002). Moreover, NG2 proteoglycan seems to play a crucial role in cell migratory properties during neuro- and gliogenesis and glial-neuron interaction (Stegmuller et al., 2002; Aguirre and Gallo, 2004; Aguirre et al., 2005; Karram et al., 2005). Cell lineage development in the spinal cord is regulated by a tightly controlled sequence of transcription factors, growth factors, adhesion and guidance molecules, as well as their corresponding receptors. The spatial distributions of BMPs and Shh seem to be major determinants of dorso-
ventral patterning of the spinal cord (Morales et al., 2006). The expression pattern of megalin as well as its specific ligand binding properties (see below) suggests an important role of this molecule in spinal cord development.

Megalin has a well established and critical function as an endocytotic, scavenger-like receptor in absorptive epithelia for low molecular weight proteins, lipids or vitamins (Hjalm et al., 1996; Christensen and Birn, 2002; Muller et al., 2003; Biemesderfer, 2006). The developmental abnormalities in the spinal cord associated with megalin deficiency, may emerge as a result of dysfunctions in these endocytotic activities of megalin. Specifically, these dysfunctions might affect the direct or carrier mediated uptake of e.g. retinol binding protein, lipids, cholesterol, and/or vitamins (e.g. vitamin D, B12) (Lin and Scanlan, 2005; Andreassen, 2006; Birn, 2006).

Since we previously identified megalin expression in immature astrocytes in the embryonic spinal cord (Wicher et al., 2005), the astroglial lineage appeared to be the most likely one to be affected in megalin deficient mice. In contrast to wild type mice, only few and weakly labeled GFAP positive astrocytes were observed at the end of prenatal development. This finding suggest that the maturation of astrocytes or a subpopulation of astrocytes is substantially delayed in megalin(-/-) mice, and/or that the population of mature astrocytes is permanently reduced. Previous studies have shown that LIF and BMP family members through activation of the JAK/STAT pathway and SMAD, respectively, directs neural stem/progenitor cells to distinct GFAP positive cell populations of astrocytes (Bonaguidi et al., 2005). LIF signalling was found to generate proliferative, bipolar/tripolar GFAP-positive cells with stem/progenitor cell properties, whereas BMP signalling generated stellate, GFAP-positive astrocytes that lack stem/progenitor cell potential (Bonaguidi et al., 2005). Disturbance in BMP signalling has previously been proposed as a mechanism underlying the abnormal forebrain development in megalin(-/-) mice (Spoelgen et al., 2005). Therefore, absence of megalin in the early stages of development may particularly influence the differentiation of astrocytes requiring BMP whereas the LIF-dependent population of astrocytes may be unaffected. Megalin may influence early CNS development also through direct and indirect interactions with morphogens and/or HSPGs (reviewed in (Fisher et al., 2006)).

BMPs and Shh are well established morphogens in the embryonic spinal cord and also ligands of megalin (McCarthry and Argraves, 2003). BMPs are produced in the roof plate, and subsequent BMP binding to megalin expressing cells may contribute to directing their development to the astroglial lineage. On the other hand, Shh, which is produced in the floor plate, plays a crucial role in promoting the development of motor neurons and oligodendrocytes in the ventral spinal cord and at the same time restricts the ventral extension of astroglial precursors (Agius et al., 2004). Therefore,
absence of megalin from developing astrocytes may not only influence differentiation and maturation of astrocytes, but also the development of oligodendrocyte precursor cells and their maturation of oligodendrocytes. Our findings that Olig2 as well as NG2 expressing cells are completely absent in the late embryonic spinal cord of megalin(-/-) mice support the presence of such astro-oligodendroglial interactions.

Comparison with previous studies reveal certain phenotypic similarities between megalin(-/-) mice and mutants for critical spinal cord morphogens and their regulators. Animals with a single mutation for BMP4(+/−) or a double mutation for the BMP antagonist Noggin(−/−) in combination with a single mutation for BMP4(+/−) display multiple malformations in the cranio-facial region as well as in the caudal spinal cord, similar to megalin mutant mice (Wijgerde et al., 2005). Moreover, megalin(-/-) mice share the absence of Olig2 positive cells in the embryonic spinal cord with Shh mutant mice (Oh et al., 2005). These similarities between megalin(-/-) mice and mice with disrupted BMP4 or Shh signaling suggest that these molecules cooperate to achieve proper differentiation of spinal cord glial cells.
Our findings emphasize a distinct neuroprotective role of clusterin following motor neuron injury, and provide evidence for a role of one of its receptors, megalin, in spinal cord development and function. Although a neuroprotective role of clusterin might be expected in view of its previously demonstrated chaperone properties, an unequivocal survival promoting effect on injured motor neurons has not been demonstrated previously. The mechanisms underlying this effect are unclear, however, and a subject for our continued investigations. In addition, it is unclear whether clusterin is also neuroprotective in spinal cord or brain injury, and in neurodegenerative disorders. Preliminary data from brain contusion injuries, suggest that clu(-/-) and clu(+/-) mice show similar extent of brain damage. Possibly, the neuroprotective effect of clusterin alone is not sufficient to counteract the wave of secondary injury processes, which operate in severe brain contusion injury.

The neuroprotective properties of clusterin seem to be exerted most efficiently by its extracellular rather than intracellular activities as indicated by our findings in clusterin overexpressing mice and in vitro. The greater neuronal network complexity after treatment with TAT-clusterin suggests that clusterin may also promote growth of neuronal processes. If further studies confirm that clusterin exerts a neuroprotective effect largely via its extracellular activities, intrathecal or systemic administration of e.g. TAT-clusterin could be considered as a potential neuroprotective therapeutic agent in selected conditions of CNS injury or disease. Further experiments with e.g. hypoxic/anoxic neuronal cultures may help to clarify the mechanisms of the neuroprotective actions of clusterin.

The selective expression of megalin in the postnatal spinal cord white matter oligodendrocytes is in sharp contrast to the expression pattern in the brain. This finding emphasizes that oligodendrocytes are regionally specialized, and that megalin has distinct roles in the physiology of spinal cord myelinated pathways. The consistent presence of the cytoplasmic tail of megalin within oligodendroglial nucleus, suggests that one of these roles is to transfer signals from the cell membrane of oligodendrocytes to their nuclei. Megalin therefore most likely plays a role as a signalling molecule, in addition to its commonly expressed function as a scavenger receptor. This interpretation receives further support by the identification of presenilin-1, which mediates endodomain cleavage of megalin, in spinal cord oligodendrocytes. Since the spatial and temporal pattern of megalin expression closely correlates with myelination of spinal cord white matter tracts, it is conceivable that megalin also plays an important role during remyelination after injury and demyelinating disorders (e.g. multiple...
sclerosis). In order to understand the function of megalin in spinal cord oligodendrocytes it is necessary to identify the ligands that induce signalling following megalin binding and the specific events during myelin formation that are regulated by this signalling.

We did not observe any change in megalin expression in the various injury models (peripheral nerve, dorsal root or direct spinal cord injury) examined. However, our studies were restricted to a survival time of one week, which may be too short to reveal any participation of megalin in e.g. delayed demyelination. Further studies, using longer postlesion survival times are necessary in order to elucidate the possible role of megalin in white matter degeneration, demyelination and repair.

A transient prenatal expression of megalin in a dorsally located subset of immature astrocytes also appears to be in contrast to current data on the localization of megalin in the brain. Although megalin has been reported to occur in the embryonic brain, the localization appears to be primarily in the ventral telencephalon. Megalin in the dorsal spinal cord may act as a local scavenger receptor to sequester the morphogens BMPs located dorsally and Shh located ventrally, thereby contributing to drive glial precursors along the oligodendroglial lineage ventrally and astroglial lineage dorsally. The failure of astrocytes to develop normally in megalin deficient mice indicates that this receptor plays a critical role for proper differentiation of these cells.

However, the finding of the cytoplasmic tail of megalin in astroglial nuclei, suggests that megalin acts not only as a scavenger receptor, but also as a signalling molecule from the cell membrane to the nucleus. The requirement for megalin expression in immature astrocytes appears to have a profound influence on oligodendroglial differentiation at later development stages, when megalin expression already disappeared. In the absence of differentiated astrocytes, oligodendroglial development appears to be interrupted prematurely, since almost no Olig2 or NG2 expressing cells were found at E16 and E20 in megalin deficient mice.

These findings highlight an important role of megalin in spinal cord development, and previous studies have demonstrated the crucial role of megalin for normal forebrain development (Willnow et al., 1996). Complete absence of megalin is not compatible with survival after birth. However, it is conceivable that minor changes in the transcription, translation and posttranslational processing of megalin may lead to dysfunction of oligodendrocytes and disturbances in myelination during pre- and postnatal development. Studies on the expression pattern of megalin in the pre- and postnatal human CNS may therefore contribute to our understanding of developmental disturbances of the brain and spinal cord.
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