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# ADHESION MOLECULES AND SYNAPSE REMODELING DURING MOTONEURON REGENERATION

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*To Stina*



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*But as we have said before, it is very rare indeed to have the good fortune of starting out with a promising study that actually produces an important discovery, and no wise investigator counts very much on doing so.*

**Santiago Ramón y Cajal**, 1897

*You can't always get what you want*
*But if you try sometimes well you just might find*
*You get what you need*

**Mick Jagger and Keith Richards**, 1968

# ABSTRACT

Spinal motoneurons integrate a vast synaptic input and are the final conveyors of motor commands to skeletal muscle. Motoneuron regeneration includes altered contacts between the motoneuron and several other cell types. The severed axon elongates along Schwann cells in the nerve, while other types of glia interact closely with lesioned motoneurons in the spinal cord. Simultaneously, cell contacts are disrupted when synapses are lost from lesioned motoneurons in a process called synaptic stripping.

The aim of this thesis project was to identify potential mediators of cell interactions during motoneuron regeneration, with special emphasis on molecules that could be of importance for synaptic remodeling. A reduced expression of cell adhesion molecules has been speculated to be involved in synaptic stripping of motoneurons. Over the last years, several such molecules have been described which influence formation and maintenance of synapses, and also mediate adhesion in other events that occur during motoneuron regeneration, such as axon guidance and myelination. We demonstrate that peripheral axotomy of sciatic motoneurons results in altered expression of several cell adhesion molecules, many of which are previously unstudied in this context. Some of these changes indicate possible involvement of the molecules in synapse elimination, whereas other molecules may be involved in other regenerative events.

Specifically, the expression of nectin-1 and -3 increased in lesioned motoneurons, as did the expression of nectin-3-binding necl-5. Nectin proteins did not localize to synapses on spinal motoneurons, but instead to neuronal processes and glia, both within the spinal cord and in the lesioned sciatic nerve. Immunoreactivity for N-cadherin localized to synapses on the surface of motoneurons and was reduced after sciatic nerve transection (SNT). Motoneuron expression of mRNA encoding N-cadherin was not altered after axotomy, and immunoreactivity for the molecule increased in the severed nerve. Axotomy also resulted in altered expression of SynCAM3/necl-1 and SynCAM4/necl-4 in the nerve, which indicates possible involvement of these molecules in remyelination. SynCAM1/necl-2, SynCAM2/necl-3, and neuroligin (NLG) -2 and -3 were expressed by unlesioned motoneurons, and SynCAM immunoreactivity localized to synapses on motoneuron cell bodies. *In vitro* these molecules have synapse-inducing properties, and following SNT, expression of SynCAM1 and NLG2 and -3 decreased rapidly, prior to loss of staining for synaptophysin in the motoneuron pool. SynCAM1 expression correlated to loss and return of synapses in regeneration after SNT. NLG expression decreased to a smaller degree after sciatic nerve crush than after SNT, although the loss of synapses was similar in both lesion models. Finally, while this work was ongoing, complement-tagging of CNS synapses for removal was demonstrated to occur in the visual system. We investigated whether complement could be involved also in synapse removal from axotomized motoneurons. Complement $C3^{-/-}$ mice displayed reduced synaptic stripping after lesion, a larger upregulation of growth-associated protein 43 in motoneurons, and a more rapid restoration of motor function.

We conclude that the motoneuron response to axotomy involves downregulation of several synaptic adhesion molecules. Expression of SynCAM1 correlates closely to the loss and return of synapses but the magnitude of the downregulation of NLGs does not seem to reflect the magnitude of the loss of synapses. Contact with the distal nerve stump may stimulate expression of NLGs, but does not seem to influence that of SynCAM1. Expression of NLGs and SynCAM1 does not seem to be the sole determinant of the elimination of synapses, since the expression pattern of these molecules was similar in mice with altered synaptic stripping and wild type mice. We also conclude that complement C3 is required for normal synapse elimination. Thus, complement may be a potential target in therapeutic attempts to preserve synaptic circuits.

# LIST OF PUBLICATIONS

This thesis is based on the following publications and manuscripts.

I. **Zelano J**, Wallquist W, Hailer NP, Cullheim S. Expression of nectin-1, nectin-3, N-cadherin, and NCAM in spinal motoneurons after sciatic nerve transection. Experimental neurology, 2006 Oct;201(2):461-9

II. **Zelano J**, Wallquist W, Hailer NP, Cullheim S. Down-regulation of mRNAs for synaptic adhesion molecules neuroligin-2 and -3 and SynCAM1 in spinal motoneurons after axotomy. Journal of Comparative Neurology, 2007 Jul, 10;503(2):308-18

III. **Zelano J**, Plantman S, Hailer NP, Cullheim S. Altered expression of nectin-like adhesion molecules in the peripheral nerve after sciatic nerve transection. Neuroscience Letters, 2009 Jan 2;449(1):28-33

IV. **Zelano J**, Berg A, Thams S, Hailer NP, Cullheim S. SynCAM1 expression correlates to restoration of central synapses on spinal motoneurons after two different models of peripheral nerve injury. Journal of Comparative Neurology, 2009. *In press*

V. Berg A*, **Zelano J***, Thams S, Pekny M, Pekna M, Cullheim S. Reduced loss of synapses on spinal motoneurons parallels more rapid motor recovery after sciatic nerve lesion in complement C3 deficient mice. *Manuscript* *equal contribution

# CONTENTS

# 1 LIST OF ABBREVIATIONS

| | |
|---|---|
| BDNF | Brain-derived neurotrophic factor |
| CNS | Central nervous system |
| EM | Electron microscopy |
| GAP-43 | Growth-associated protein 43 |
| GFAP | Glial fibrillary acidic protein |
| GluR1 | Glutamate receptor 1 |
| IHC | Immunohistochemistry |
| IL/CL | Ipsilateral/contralateral |
| ISH | In situ hybridization |
| mEPSCs | Miniature end plate synaptic currents |
| MHC | Major histocompatibility complex |
| mRNA | Messenger ribonucleic acid |
| N-cadherin | Neural-cadherin |
| NCAM | Neural cell adhesion molecule |
| Necl | Nectin-like molecule |
| NLG | Neuroligin |
| NMDA | *N*-methyl-*D*-aspartic acid |
| NT-3 and -4 | Neurotrophins-3 and -4 |
| PBS | Phosphate buffered saline |
| PNS | Peripheral nervous system |
| PSA | Polysialic acid |
| PSD-95 | Postsynaptic density 95 |
| RNAi | RNA interference |
| SNC | Sciatic nerve crush |
| SNT | Sciatic nerve transection |
| SSC | Standard saline citrate |
| SynCAM | Synaptic cell adhesion molecule |
| VAChT | Vesicular Acetylcholine transporter |
| Vglut2 | Vesicular glutamate transporter 2 |
| Viaat | Vesicular Inhibitory Amino Acid Transporter |
| WT | Wild type |

# 2 INTRODUCTION

## 2.1 GENERAL INTRODUCTION

### 2.1.1 Spinal motoneurons

Spinal motoneurons extend from the central nervous system (CNS) into the peripheral nervous system (PNS). The cell bodies of motoneurons reside in the ventral horn of the spinal cord and extend extensive trees of dendrites on which each neuron receives tens of thousands of synapses (Ulfhake and Cullheim, 1988). These interneuronal connections provide information from higher levels of motor control and from sensory neurons in reflex circuits (Puskar and Antal, 1997). The axons of spinal motoneurons extend from the motoneuron cell bodies to skeletal muscle through peripheral nerves, in which the axons are insulated by Schwann cells. In summary, spinal motoneurons integrate a vast synaptic input and transmit motor commands to skeletal muscle.

Axons from motoneurons in several spinal cord segments are sorted into peripheral nerves at plexuses in the cervical and lumbar regions. From these plexuses, peripheral nerves extend to muscles in the limbs. The studies in this thesis have been performed on sciatic motoneurons, which are situated in an elongated nucleus at the lower lumbar level of the spinal cord and whose axons travel through the sciatic nerve to the lower limb (Swett et al., 1986).

### 2.1.2 Synapses

Synapses in the CNS are junctions between nerve cells, specialized in transmission of signals from one nerve cell to another, as described in Kandel (2000). Briefly, a specialized structure of the presynaptic neuron, the terminal, is closely fixed to a specialized region of the postsynaptic neuron. The terminal contains synaptic vesicles filled with neurotransmitter molecules. Upon altered membrane potential, the synaptic vesicles fuse with the membrane surface facing the postsynaptic neuron and neurotransmitter molecules are emptied into the synaptic cleft, where they diffuse to the postsynaptic surface and interact with receptors.

The pre- and postsynaptic membranes of synapses are held together by adhesion molecules, which extend from the pre- and postsynaptic surfaces. Over the last years, these adhesion molecules have proven to be more than mere hooks which maintain the structural integrity of the synapse. In fact, expression of certain adhesion molecules seem to instruct other neurons to form synapses and decreased expression levels of these molecules seem to reduce synaptic inputs, as reviewed in Biederer (2006) and Brose (2009). Thereby, expression of postsynaptic adhesion molecules offers a possible mechanism by which neurons could alter their synaptic input.

### 2.1.3 Motoneuron injury

Axotomy of a spinal motoneuron is followed by reactions in both the PNS and the CNS. The injured motoneuron itself undergoes profound changes in order to regenerate, but other cell types such as Schwann cells, astrocytes, microglia, macrophages, and muscle cells also change their gene expression and behaviour during restoration of normal function.

In the PNS, the regenerative efforts are focused on clearing the isolated distal part of the axon and paving the way for a new, regenerating one. The distal segment of the axon is degraded in a process called Wallerian degeneration (Waller, 1850). Macrophages infiltrate the nerve and clear debris together with Schwann cells (Perry et al., 1987; Reichert et al., 1994; Stoll et al., 1989). Simultaneously, the latter proliferate and guide regenerating axons (Cheng and Zochodne, 2002; Son and Thompson, 1995). In the proximal stump of the severed nerve, the axon end transforms into a growth cone, a neuronal structure that is seen during development (Kandel et al., 2000). The growth cone senses cues in the environment and then grows in the direction deemed appropriate. As the axon grows along the Schwann cells, the latter begin to produce myelin and remyelinate the axon. Axonal regeneration is reviewed in Fawcett and Keynes (1990).

Within the CNS, peripheral axotomy elicits changes in motoneuron gene expression and morphology, but also a response by glia. Within days, astrocytes and microglia are activated and interact closely with the lesioned motoneurons (Blinzinger and Kreutzberg, 1968; Graeber et al., 1988; Reisert et al., 1984; Tetzlaff et al., 1988) which in turn display a switch from a transmitting phenotype to a regenerating one. This switch involves increased expression of proteins important for regeneration, such as growth-associated protein 43 (GAP-43), and decreased expression of proteins important for transmission, such as choline acetyltransferase (ChAT) and NMDA-receptor subunits (Davidoff and Schulze, 1988; Lindå et al., 1992; Piehl et al., 1995b). Functionally, the lesioned motoneuron displays reduced monosynaptic inputs (Eccles et al., 1958), as a consequence of a process called synaptic stripping, by which synapses on the dendrites and soma of the motoneuron are lost (Blinzinger and Kreutzberg, 1968; Chen, 1978; Sumner, 1975a). Microglia, astrocytes, and major histocompatibility complex (MHC) class I proteins have all been implicated in this process (Blinzinger and Kreutzberg, 1968; Oliveira et al., 2004; Reisert et al., 1984; Svensson and Aldskogius, 1993), but the exact mechanisms underlying the elimination of synapses from lesioned motoneurons are not known (Aldskogius et al., 1999). The removal of synapses has been suggested to be beneficial to regenerating motoneurons, by protecting the cells from excitatory input and allowing the allocation of resources to regeneration rather than transmission (Lindå et al., 2000; Svensson and Aldskogius, 1993).

In summary, peripheral axotomy of motoneurons offers an accessible lesion model that, in addition to studies on peripheral nerve regeneration, can have bearing on several pathological conditions in the nervous system. Since the regenerative capacity of peripherally axotomized spinal motoneurons is vastly superior to that of neurons which have been axotomized within the CNS, comparative studies strive to determine differences between peripheral and central regeneration and thereby identify conditions and mechanisms associated with regenerative success. In addition, several processes that occur during motoneuron regeneration, such as CNS inflammation, myelination, axon guidance, and loss of CNS synapses, are also features of other pathological conditions in the nervous system.

### 2.1.4 Cell-cell adhesion molecules in motoneuron regeneration

Complex signalling is required to orchestrate motoneuron regeneration. Briefly, the motoneuron interacts with other cells by several types of signalling; small factors such

as neurotrophins are secreted from glia and muscle cells and diffuse to neuronal receptors (reviewed in Terenghi, 1999), other molecules such as laminins are deposited in the extracellular environment and detected by neuronal receptors (reviewed in Lemons and Condic, 2008), and finally, regenerating motoneurons are in direct contact with other cells by membrane cell-cell adhesion molecules, such as neural cell adhesion molecule (NCAM) and neural (N)-cadherin (reviewed in Martini, 1994).

Since the challenges for an axotomized neuron in adulthood in many ways resemble those facing a growing neuron in development, an often used approach to the study of neuronal regeneration has been to examine whether molecular systems that are important during development are also mobilized after injury. Over the last years, several new cell-cell adhesion molecules have been discovered, such as nectins, neuroligins, neurexins, and SynCAMs/nectin-like adhesion molecules. During development, these molecules are involved in cell-cell interactions such as axon guidance, myelination, and adhesion in synapses – processes that are relevant also in adult motoneuron regeneration. However, the expression of the above listed molecules in regenerating motoneurons had, prior to the studies in this thesis, not been addressed.

## 2.2 ADHESION MOLECULES INVESTIGATED IN THIS THESIS

Studies on cell-cell adhesion molecules in axonal regeneration have largely been focused on peripheral regenerative events, such as contacts between regenerating axons and the extracellular matrix or Schwann cells (Martini, 1994). Given the recently discovered importance of cell-cell adhesion molecules in synaptic biology, we decided to investigate adhesion molecules that could be of importance for adhesion in synapses on spinal motoneurons. Synaptic stripping of lesioned motoneurons must at some point involve a disruption of the adhesion between pre- and postsynaptic membranes and decreased affinity between the membranes, perhaps due to decreased expression of postsynaptic adhesion molecules, has been speculated to be involved in the removal of synapses (Aldskogius et al., 1999; Sumner, 1975b). The list of candidates included several recently described molecules, but also the well known NCAM and N-cadherin, which are involved in motoneuron regeneration and have synaptic roles elsewhere in the CNS (see below). In addition to a search for expression patterns that could suggest involvement of investigated molecules in synaptic adhesion, our studies were expanded when the results pointed to interesting roles for a candidate molecule in other regenerative events.

### 2.2.1 Nectins

Nectins are a family within the immunoglobulin superfamily with four members, nectin-1 through -4. Nectins colocalize with cadherins and the recruitment of nectins to sites of cadherin adhesion depend on binding of nectins to afadin, an actin-filament binding protein (Sakisaka and Takai, 2004; Takahashi et al., 1999). Nectins interact in trans both homo- and heterophilically (Satoh-Horikawa et al., 2000).

During synaptogenesis, nectins colocalize with N-cadherin in synapses in the hippocampus, with nectin-1 and -3 localized to the pre- and postsynaptic membranes, respectively (Mizoguchi et al., 2002). The role of nectins in synaptic adhesion is not clear. Administration of a nectin-inhibitor results in smaller synapse size and an increase in synapse number on cultured hippocampal neurons (Mizoguchi et al., 2002), but nectin-$1^{-/-}$ and nectin-$3^{-/-}$ mice display normal immunoflourescence levels of

synaptic markers such as Basoon, postsynaptic density 95 (PSD-95), and GluR1 in the hippocampus, as well as normal synaptic transmission, indicating that nectin interaction is not essential for the formation of synapses (Honda et al., 2005).

Nectin-1 and -3 are expressed in the developing nervous system during embryogenesis and mediate contacts between growing neurites and the commissural plate (Okabe et al., 2004a; Okabe et al., 2004b). Nectin-$1^{-/-}$ or nectin-$3^{-/-}$ mice display an intact gross anatomy of the nervous system (Honda et al., 2005), indicating either redundancy within the nectin family, or that other mechanisms can compensate for the loss of a single nectin in the gross development of the CNS. However, nectins are most likely involved in guidance of neuronal projections, since the localization of mossy fibres in the hippocampus is altered in nectin-$1^{-/-}$ or nectin-$3^{-/-}$ mice (Honda et al., 2005). Also supportive of a role for nectins in formation of interneural connections are findings that nectin-1 localizes to axons and nectin-3 to both axons and dendrites, and that modified expression of nectin-1 alters the formation of axo-dendritic contacts *in vitro* (Togashi et al., 2006).

Some information exists on the expression of nectins in the spinal cord. Nectin-1 protein is expressed in peripheral sensory neurons and a low expression has been described in spinal motoneurons (Mata et al., 2001), which fits well with a report on expression of nectin-1 mRNA in this cell type (Haarr et al., 2001).

### 2.2.2 SynCAMs / nectin-like molecules

Synaptic cell adhesion molecules (SynCAMs) belong to the immunoglobulin superfamily and make up a family of four adhesion molecules, SynCAM1-4, so named because SynCAM1 was identified in a synaptic context (Biederer et al., 2002). The molecules were simultaneously identified by other research groups, and accordingly received many different names. One proposed nomenclature refers to the molecules as nectin-like (necl) molecules, because of their structural similarity to nectins (Ikeda et al., 2003). The necl family consists of five members – necls-1 through necl-5. Currently, no consensus on nomenclature has been reached. In this thesis, the name SynCAM was used in papers II and IV, where the molecules were discussed in a synaptic context, and the name necl in paper III where their possible roles in neuron-glia interactions were the main focus. In this summary, the names are used interchangeably as deemed fit by the context, but the molecules are introduced in their SynCAM-order with dual names.

SynCAM1/necl-2 mediates adhesion both homophilically, and heterophilically with SynCAM2/necl-3, SynCAM3/necl-1 and nectin-3 (Fogel et al., 2007; Shingai et al., 2003). SynCAM1/necl-2 localizes to both the pre- and postsynaptic membrane in synapses (Fogel et al., 2007), and overexpression of SynCAM1/necl-2 or expression of a dominant negative variant of the molecule causes increased or decreased synaptic input to cultured neurons, respectively (Biederer et al., 2002; Sara et al., 2005). Furthermore, expression of SynCAM1/necl-2 in non-neuronal cells causes co-cultured hippocampal neurons to form synapses on the SynCAM1/necl-2-expressing cells (Biederer et al., 2002). Initially, SynCAM1/necl-2 was perceived to mediate homophilic interactions between synaptic membranes, but with increasing knowledge on SynCAMs, it seems that other SynCAMs may also have synaptic functions and that family members interact with each other. This notion is supported by findings of Fogel and co-workers that all SynCAMs are present in synaptic membranes in postnatal rat

forebrains, that SynCAM1/necl-2 interacts strongly with SynCAM2/necl-3, that these molecules interact in synapses *in vivo*, and that both molecules have the capacity to recruit presynaptic proteins (Fogel et al., 2007). SynCAM1/necl-2 expression is dynamic during formation of synaptic contacts in the spinal cord and during a period of synaptic rearrangement in the visual cortex, indicating roles for the molecule in formation of neuronal circuits *in vivo* (Lyckman et al., 2008; Thomas et al., 2008).

SynCAM2/necl-3 mediates homophilic adhesion as well as heterophilic adhesion with SynCAM1/necl-2, SynCAM3/necl-1, or SynCAM4/necl-4. In addition to its synaptic location, SynCAM2/necl-3 is present on the surface of myelinated axons in the CNS, where it is distributed to the interface between oligodendrocytes and axons. SynCAM2/necl-3 seems to have a ligand on oligodendrocytes, since SynCAM2/necl-3-coated surfaces retain oligodendrocytes but not most other cell types in mixed primary cultures from the cerebellum (Pellissier et al., 2007).

SynCAM3/necl-1 is mainly expressed in the nervous system. In the CNS it localizes to contact sites between presynaptic nerve terminals, axons, and glial cell processes, and in the PNS to contact sites between Schwann cells at the nodes of Ranvier. SynCAM3/necl-1 mediates homophilic adhesion as well as heterophilic adhesion with SynCAM1/necl-2, SynCAM2/necl-3, SynCAM4/necl-4, nectin-1, and nectin-3 (Kakunaga et al., 2005; Spiegel et al., 2007).

SynCAM4/necl-4 is expressed by Schwann cells at the onset of myelination, and mediates Schwann cell-axonal adhesion by binding to axonal SynCAM3/necl-1. The interaction between SynCAM3/necl-1 and SynCAM4/necl-4 is important for myelination since treatment with soluble SynCAM4/necl-4 inhibits remyelination of demyelinated sciatic nerve *in vivo* and since disruption of SynCAM3/necl-1-SynCAM4/necl-4 interaction by soluble forms of the molecules as well as RNAi of SynCAM4/necl-4 inhibits myelination in cultures of DRG-neurons and Schwann cells (Maurel et al., 2007; Spiegel et al., 2007).

Necl-5 regulates cell-movement and proliferation (Ikeda et al., 2004; Kakunaga et al., 2004). Necl-5 interacts heterophilically with nectin-3 (Ikeda et al., 2003) and upon necl-5-nectin-3 interaction, necl-5 is downregulated from the cell surface and movement and proliferation of cultured cells is reduced (Fujito et al., 2005).

### 2.2.3 NCAM

NCAM was one of the first adhesion molecules to be identified in neurons (Rutishauser et al., 1976). NCAM is a member of the immunoglobulin superfamily and contains six glycosylation sites. Two of these sites can be glycosylated with polysialic acid (Kruse et al., 1984), which is believed to reduce the strength of NCAM-mediated adhesion (Yang et al., 1992).

NCAM has many roles in the nervous system. NCAM interacts with components of the microtubule cytoskeleton, which is involved in neurite extension (Buttner et al., 2003). In peripheral nerves, NCAM mediates interactions between axons and Schwann cells (Martini and Schachner, 1986; Martini and Schachner, 1988; Nieke and Schachner, 1985; Seilheimer et al., 1989) and NCAM immunoreactivity increases in transected peripheral nerves (Daniloff et al., 1986; Martini and Schachner, 1988; Thornton et al., 2005), where NCAM expression is stimulated by neurotrophin administration (Thornton et al., 2008). Recent evidence support an important role for NCAM in axon guidance in motoneuron regeneration, since NCAM/PSA$^{-/-}$ mice, and

WT mice where PSA is enzymatically removed, lose the selective targeting of regenerating motor axons (Franz et al., 2005).

NCAM is involved in the formation and function of synapses. NCAM-expressing cultured neurons display increased synaptic activity and more synapses are formed on NCAM-expressing neurons than on co-cultured non-NCAM-expressing ones (Dityatev et al., 2000). *In vivo*, PSA and NCAM are important for correct synaptogenesis in the hippocampus (Seki and Rutishauser, 1998) and $NCAM^{-/-}$ mice exhibit impaired memory and reduced long-term potentiation (LTP) (Cremer et al., 1998; Cremer et al., 1994).

### 2.2.4 N-cadherin

N-cadherin was identified in 1982 (Grunwald et al., 1982; Lagunowich and Grunwald, 1989). In the nervous system, N-cadherin mediates adhesion between neurons and between neurons and glia (Tomaselli et al., 1988) and the molecule is involved in neurite outgrowth and axon guidance (Benson and Tanaka, 1998; Nakai and Kamiguchi, 2002).

Together with NCAM, N-cadherin was one of the first adhesion molecules to be implicated in axon regeneration. Interactions between axons and Schwann cells can be mediated by N-cadherin (Bixby et al., 1988) and the molecule is expressed both by regenerating axons and Schwann cells (Cifuentes-Diaz et al., 1994). N-cadherin is present on growth cones *in vitro* (Letourneau et al., 1990) and expression of the molecule is increased in severed nerves (Shibuya et al., 1995; Thornton et al., 2005), where locally administered NT-4 increases N-cadherin expression at the regeneration front and NT-3 administration results in decreased N-cadherin levels (Thornton et al., 2008).

Many studies also indicate a role for N-cadherin in synapse formation. The molecule is localized to synapses (Benson and Tanaka, 1998; Fannon and Colman, 1996; Huntley and Benson, 1999) and N-cadherin has been implicated in synaptogenesis and synaptic plasticity induced by injury (Bamji et al., 2003; Benson and Tanaka, 1998; Brock et al., 2004; Fannon and Colman, 1996; Jontes et al., 2004; Prakash et al., 2005; Shan et al., 2002; Yamagata et al., 1995). In synapse formation, N-cadherin interacts with other adhesion molecules such as nectins, through common intracellular binding proteins (Mizoguchi et al., 2002; Takahashi et al., 1999). One proposed model suggests that N-cadherin-mediated adhesion follows initial adhesion by nectins in the formation of synapses (Takai et al., 2003).

N-cadherin is expressed by spinal motoneurons (Eleore et al., 2005; Monks and Watson, 2001) and axotomy induces decreased β-catenin (an intracellular binding partner of N-cadherin) and pan-cadherin immunoreactivity on the surface of motoneurons in the facial nucleus (Eleore et al., 2005).

### 2.2.5 Neuroligins

NLGs are synaptic adhesion molecules that localize to postsynaptic membranes and were discovered as ligands for the presynaptic neurexins (Berninghausen et al., 2007; Ichtchenko et al., 1995; Ichtchenko et al., 1996; Song et al., 1999). Neurexins and NLGs assemble pre- and postsynaptic specializations, respectively, and transsynaptic interaction between the molecules induces synapse formation in various assays (Dean et al., 2003; Graf et al., 2004; Nam and Chen, 2005; Scheiffele et al., 2000).

Structurally, neuroligins belong to the CLAMs (cholinesterase-like adhesion molecules), whose extracellular domain resembles that of cholinesterases (Gilbert and Auld, 2005). Intracellularly, NLGs bind to postsynaptic proteins such as PSD-95 (Irie et al., 1997).

NLGs display selectivity in their distribution. NLG1 localizes primarily to excitatory synapses and NLG2 localizes primarily to inhibitory ones (Graf et al., 2004; Levinson et al., 2005; Song et al., 1999; Varoqueaux et al., 2004), whereas NLG3 localizes to both types of synapses (Budreck and Scheiffele, 2007). Overexpression of NLGs induces an increased number of synapses (Chih et al., 2005; Levinson et al., 2005; Prange et al., 2004) and RNAi of NLG1-3 reduces excitatory and inhibitory synapse formation *in vitro*, with the largest response for inhibitory synapses seen when expression of NLG2 is suppressed (Chih et al., 2005). Recently, the issue has been raised whether NLGs might act through other mechanisms than binding of presynaptic neurexins (Ko et al., 2009), but nonetheless, just like SynCAM1, neuroligins offer another possible mechanism by which a neuron could regulate its afferent connections.

Interestingly, knock-out of NLGs in mice does not lead to a reduction of synapse number, but an altered synaptic function (Poulopoulos et al., 2009; Varoqueaux et al., 2006). This prompted further investigations into the exact role of NLGs in synapses and the increase in synapse number seen when NLGs are overexpressed *in vitro* can be blocked by inhibitors of synaptic activity, indicating that NLGs do not initiate formation of new synapses but are involved in an activity-dependent synapse maturation (Chubykin et al., 2007). These results are in line with the finding that cultured neurons in which endogenous NLG1 expression is suppressed display a decreased number of mEPSCs, but not fewer synaptic puncta (Conroy et al., 2007). Thus, it is possible that neuroligins are not required for the formation of synapses *per se*, but are required for proper synaptic function (Südhof, 2008; Varoqueaux et al., 2006).

NLGs have also attracted interest because of their possible involvement in autism spectrum disorders. Mutations in the genes encoding NLG3 and NLG4 have been implicated in hereditary autism (Jamain et al., 2003; Laumonnier et al., 2004; Zhang et al., 2009) and several reports now exist on autistic-like behaviour and altered synaptic function in animals with altered NLG expression. Introduction of one of the human autism linked mutations in mice was reported to cause altered synaptic function and enhanced spatial learning abilities (Tabuchi et al., 2007), although these findings were not repeated in an independently developed mouse strain with the same mutation (Chadman et al., 2008). However, behavioural deficits have now been described in mice with mutations in or altered expression of either of NLG2-4 (Blundell et al., 2009; Hines et al., 2008; Jamain et al., 2008; Radyushkin et al., 2009) and silencing of NLG1 in the amygdale of rats *in vivo* leads to a reduction of LTP and associative fear memories (Kim et al., 2008). Taken together, these reports indicate that altered NLG expression causes altered synaptic function *in vivo* and altered behaviour.

## 2.3 THE COMPLEMENT SYSTEM IN MOTONEURON REGENERATION

The complement system consists of proteins circulating in the blood and tissue. Upon recognition of foreign invaders, the complement system is activated and through a series of cleavages produces factors that attract inflammatory cells, mark foreign material for phagocytosis, and lyse foreign cell membranes. The traditional role of the

complement system is defence against invading micro organisms, but complement is also involved in neurotrauma-induced inflammation (Bellander et al., 1996).

The complement system is upregulated in the peripheral nerve after nerve lesions, and required for recruitment of macrophages into the injured nerve for Wallerian degeneration (Bruck and Friede, 1990; Dailey et al., 1998; Ramaglia et al., 2007). Complement is also expressed within the spinal cord after peripheral nerve lesions (Liu et al., 1995; Mattsson et al., 1998; Svensson and Aldskogius, 1992; Svensson et al., 1995). While the work on this thesis was ongoing, a new role for complement was described in synaptic biology, in that complement deficient animals display a decreased removal of synapses in the visual system during development and retinal degeneration (Stevens et al., 2007). Based on these findings and on the fact that certain complement factors are known to be upregulated in the vicinity of axotomized motoneurons, we investigated the role of complement in synaptic stripping of adult motoneurons.

# 3 AIMS

The general aim of this PhD-project was to investigate possible new mediators involved in the response within the spinal cord to peripheral axotomy of motoneurons, specifically molecules that could be of importance for synaptic remodeling of lesioned motoneurons.

Based on the recent advances in our understanding of the importance of cell-cell adhesion molecules in synaptic biology, we hypothesised that the motoneuron response to axotomy involves downregulation of synaptic adhesion molecules and that the expression of such molecules governs the synaptic input to motoneurons.

The following specific aims were set up:

- To study the expression of candidate cell-cell adhesion molecules in spinal motoneurons, with special emphasis on molecules that could be of importance in synaptic adhesion.

- To study the subcellular localization of these cell-cell adhesion molecules.

- To study the expression of these cell-cell adhesion molecules after axonal injury and relate the expression to synaptic stripping.

- To study the expression of molecules deemed relevant in the above studies in relation to synaptic stripping after different kinds of axonal injury and also relate the expression to synapse restoration after regeneration.

- To study factors that might regulate the expression of the identified cell-cell adhesion molecules.

Our investigations were expanded when the results pointed to interesting roles for an investigated molecule in other regenerative events.

While this work was ongoing, a role was demonstrated for complement in CNS synapse elimination. We therefore hypothesised that complement C3 is required for synaptic stripping of axotomized motoneurons.

# 4 MATERIALS AND METHODS

## 4.1 SUMMARY OF EXPERIMENTAL TECHNIQUES

### 4.1.1 Animal experiments

In papers I-IV, young female Sprague-Dawley rats (B&K Universal, Sweden) with a body weight of approximately 200 g were used. Chloral hydrate (papers I and II) or a mixture of Hypnorm and Midazolam (papers III and IV) was used as anaesthesia. The rats were kept on a heating pad during surgery and given saline containing buprenorphine as fluid replacement and analgesia.

For studies on the acute response to axotomy (papers I-III), the sciatic nerve was transected (sciatic nerve transection, SNT) at mid thigh level and 7-10 mm of the nerve was resected to prevent regeneration. For studies on regeneration (paper IV) the sciatic nerve was either transected without resection or crushed (sciatic nerve crush, SNC).

After the appropriate survival time, the rats were deeply anesthetized with pentobarbital administered intraperitoneally. Animals used for in situ hybridization (ISH) were decapitated, whereas animals used for immunohistochemistry (IHC) were transcardially perfused with Tyrode's solution for 5-10 s, followed by a fixative containing 4% formaldehyde and 0.4% picric acid. Animals used for electron microscopy (EM) were transcardially perfused with fixative containing 2% glutaraldehyde in Millonig's buffer. Postnatal rats were decapitated (paper III).

In paper V, mice underwent the same surgery as the rats on Hypnorm/Midazolam anaesthesia. Instead of pentobarbital, carbon dioxide was used for euthanasia.

### 4.1.2 Behavioural analysis

For behavioural experiments (papers IV and V), the hind limb paws were painted with water-based paint and the rat (paper IV) or mouse (paper V) traversed a tunnel placed on a sheet of paper. Measurements of intermediary toe spread (distance from second to fourth toe) and toe spread (distance from first to fifth toe) were obtained from the first three foot prints and averaged (Bain et al., 1989). In paper V, we also assessed foot faults in a grid walk paradigm and grip strength. The latter was assessed by letting the animal grip a metal rod which was then slowly pulled away. The grip strength was graded according to a three step scale – no grip, insufficient grip compared to the unlesioned paw, or similar grip as the unlesioned paw.

### 4.1.3 Tissue preparation

For ISH the lumbar section of the spinal cord and/or the sciatic nerve and/or the brain were rapidly dissected. The tissues were promptly frozen and sectioned in a cryostat in 12 μm transverse sections at -20°C and stored at that temperature until used.

For IHC and EM, the lumbar section of the spinal cord and/or the sciatic nerve were rapidly dissected after perfusion. For IHC, after immersion fixation in the same fixative, the tissues were transferred into 0.01 M PBS, containing 0.1% sodium azide and stored at 4°C until one day before sectioning, when they were transferred to PBS containing 10% sucrose. For EM, the spinal cords were immersion fixed at 4°C before further processing.

In experiments where IHC was performed on tissue from animals which were also used for ISH (papers II and IV), a different fixation technique was used in which sections obtained for ISH were immersion fixed at room temperature.

For western blot, the lumbar section of the spinal cord was rapidly dissected. Two transversal sections isolated the lower 5 mm of the lumbosacral enlargement, which was then further dissected to isolate the ventral half of the spinal cord. The tissue was then sonicated in 1% sodium dodecyl sulphate and boiled for 10 minutes.

### 4.1.4 Western blot

After protein content determination (Pierce Europe), 30 μg of total protein was loaded onto 10% polyacrylamide gels, separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Amersham) and immunoblotted according to the manufacturers instructions. Antibody binding was revealed by the Odyssey detection system (Odyssey®, Licor).

### 4.1.5 In situ hybridization

Oligonucleotides were synthesized (CyberGene AB, Sweden) and ISH was performed as previously described (Dagerlind et al., 1992). The sequences of the probes were checked in a GeneBank database search to exclude significant homology with other genes. The probes were labelled at the 3'-end with deoxyadenosine-alpha-triphosphate [$^{33}$P] and hybridized to the sections without pre-treatment for 16-18 hours at 42°C. Following hybridization, the sections were washed several times in 1 x SSC at 55°C, dehydrated in ethanol, and dipped in NTB2 nuclear track emulsion (Kodak, NY, USA). After 3 weeks, the slides were developed in D-19 developer (Kodak), counterstained and coverslipped. The sections were examined in a Leica microscope (Leica, Germany).

### 4.1.6 Immunohistochemistry

Sections were incubated with antisera containing primary antibodies and normal donkey serum, rinsed in PBS, incubated with flourophore-conjugated secondary antibodies diluted in PBS and Triton X-100, rinsed in PBS and mounted in PBS-glycerol (1:3). To evaluate the degree of background staining, the primary antibody was omitted or incubated with a surplus of blocking peptide in the staining of control sections. For double labelling experiments, sections were processed as described above, with additional primary and secondary antibodies. For all double labelling experiments, control stainings were performed for each primary antibody with that antibody omitted, and single stainings were also performed to safeguard against cross reactivity.

### 4.1.7 Semiquantitative measurements of ISH signal

Semiquantitative measurements were carried out as previously described (Piehl et al., 1995a). Briefly, the mRNA hybridization signal overlaying the area of interest – motoneuron cell bodies, distal stumps of the sciatic nerve, etc, was recorded with a Leica microscope and digitized using a Kappa video camera (Mikroskop System, Sweden). The grey scale of the dark field image was adjusted and segmented using the enhance contrast and density slicing feature of the NIH Image software (version 1.55; NIH, MD, USA) so that the silver grains over relevant areas were assessed

automatically. For oligoprobes whose signal was deemed similar in all motoneurons, the mean signal over ipsilateral (IL) injured neurons was compared to that over contralateral (CL) uninjured neurons in the same spinal cord section and the average IL/CL ratio of four spinal cord sections from each animal was used for statistical analysis.

For nectin-3 (paper I), the ISH signal was distributed to a subpopulation of motoneurons in uninjured animals. We therefore measured the ISH signal over individual sciatic motoneurons and expressed the signal in relation to background. Cells with a labelling of more than five times the background, an arbitrary and perhaps somewhat conservative threshold, were considered positive. A similar approach was used for measurements of ISH signal over sciatic nerves. The signal over the stump of the nerve was related to the signal over the glass adjacent to the section.

### 4.1.8 Semiquantitative measurements of immunoreactivity

Semiquantitative measurements of immunoreactivity were performed in Scion Image® (Scion corporation, paper II and IV) or ImageJ® (NIH, paper IV and V). These softwares allow the measurement of pixels with a grey value above a user-defined threshold. For assessment of synaptic stripping (paper II, IV, and V) the immunoreactivity in the sciatic motoneuron pool was measured and compared to the signal in the same area in the contralateral side of the spinal cord, resulting in an IL/CL ratio. Similarly to the ISH measurements, the average IL/CL ratio of several spinal cord sections from each animal was used for statistical analysis. The images were taken in the optical plane with the most immunoreactivity and all settings for compared images were identical.

In paper IV, we assessed changes in SynCAM immunoreactivity. Since SynCAMs have several extrasynaptic locations and are not exclusively expressed by motoneurons, the approach with large area measurements was deemed inappropriate. Instead, we measured the SynCAM immunoreactivity on the surface of motoneurons, visualized by double labelling with antisera against synaptophysin. Measurements were obtained from three injured motoneurons in one spinal cord section from each of four injured animals, and the average signal was used for statistical analysis. Motoneurons were identified by their size and location in the sciatic motor column. The average signal of three uninjured motoneurons on the contralateral side of each section was used as paired controls. All spinal cord sections were present on the same slide and thus exposed to the same antibody solution, all images were taken in the optical plane with the maximal immunoreactivity for synaptophysin, and all settings for all images were identical.

### 4.1.9 Electron microscopy

The specimens were trimmed, osmicated, dehydrated, and embedded in plastic resin. Synaptic terminals apposed to the motoneuron cell bodies were identified and their number per 100 μm cell membrane length was calculated. In paper IV, two neurons were studied in each of three animals per survival time and lesion model, one contralateral uninjured motoneuron was studied in each of three animals as controls. In paper V, two motoneurons were studied in each of five WT and five $C3^{-/-}$ animals.

## 4.2 METHODOLOGICAL CONSIDERATIONS

### 4.2.1 In situ hybridization

In this thesis, mRNA expression is studied by radioactive ISH. Some of the most important limitations of this method concern detection of low levels of mRNA expression, interpretation of semiquantitative measurements, and probe specificity. Regarding low levels of mRNA expression, radioactive ISH is not able to exclude presence of a target mRNA, since the method always results in some background signal. Regarding semiquantitative measurements of ISH signal, the presented results constitute comparisons of ISH signals and not absolute quantifications of mRNA levels. ISH signal strength depends on many variables, for instance tissue storage time, washing temperature, and emulsion thickness. In the papers upon which this thesis is based, we only compare ISH signal over one area to signal over an adjacent area, preferably in the same tissue section. Thereby, compared areas should have been subjected to the same experimental conditions. In some cases, the signal strength has been related to the background signal over glass. This gives only an appreciation of signal strength, but a signal more than five times the background is a fairly conservative limit for considering a signal positive.

Regarding probe specificity, database searches were performed to exclude cross reactivity with other mRNAs. In addition, we used control tissues with known expression of the investigated molecules to verify the specificity of our probes. As an additional precaution against detection of erroneous mRNAs, we synthesized control probes directed against a different portion of the target mRNA and verified that these probes rendered signal patterns identical to those from the probes used for semiquantification. The approach with two probes against each mRNA was used for all investigated molecules except SynCAM2, where a shortage of tissue forced us to prioritize other experiments. However, the specificity of the SynCAM2 probe was verified by hybridization to sections of rat brain, where the signal pattern was identical to that reported by other investigators (Thomas et al., 2008).

### 4.2.2 Immunohistochemistry

IHC depend heavily on the reliability of the antibodies used (Saper, 2005). Possible control experiments include western blot, blocking peptides, staining of tissue where the expression has been previously described, and relation of observed immunoreactivity to mRNA expression. In the papers upon which this thesis is based, we have tried to use only well characterized antibodies and to apply appropriate controls.

### 4.2.3 Species differences

The first part of this thesis (papers I-IV) investigates the expression of cell-cell adhesion molecules in injured motoneurons in the rat, whereas the last paper (V) concerns motoneuron injury in mice. Anatomical differences (Rigaud et al., 2008) should not influence our results, since lesion controls such as upregulation of GAP-43 or IHC for glial reactions were used throughout all experiments. However, differences exist between mice and rats in the reaction to motoneuron axotomy. In general, an anatomically identical lesion results in a more severe reaction in the mouse. For

instance, most motoneurons survive nerve transection in the rat, but a certain cell death occurs in the mouse – for reviews see Lowrie and Vrbova (1992) and Moran and Graeber (2004). The phenomenon of synaptic stripping seems to be fairly similar in the two species (papers II, IV, and V) but, nonetheless, some caution should be exercised when extrapolating findings between them.

### 4.2.4 Electron microscopy

IHC for synaptic markers offers a rapid method for assessment of synaptic stripping and has the advantage of allowing additional investigations of material from the same animal. However, as the method is semiquantitative and measures not the number of synapses on motoneurons *per se*, but the staining intensity for a presynaptic marker, we wanted to confirm our IHC findings by EM. In paper IV and V, EM verified an elimination of synapses from injured motoneurons, and a restoration of synapses after SNC in paper IV. Generally, the synaptic stripping was larger when measured by EM than by IHC and the restoration of synapses less complete. This difference could have several explanations. First of all, we measured synaptophysin immunoreactivity in the entire sciatic motoneuron pool, which contains motoneuron cell bodies and dendrites, whereas our EM analysis was restricted to somatic terminals. The elimination of synapses is relatively more severe from the motoneuron soma and proximal dendrites (Brännström and Kellerth, 1998), although in absolute number more synapses are lost from dendrites than from cell bodies. Second, only terminals which could be identified with certainty and were in contact with the motoneuron membrane were counted in the EM experiment. Since terminals on lesioned motoneurons undergo morphological changes (Blinzinger and Kreutzberg, 1968; Chen, 1978), it is possible that our EM underestimates the number of terminals on lesioned motoneurons. To the contrary, our IHC may overestimate the number of remaining terminals. It is not known at what time immunoreactivity for synaptic markers is lost from terminals that are eliminated and we cannot exclude that some immunoreactivity surrounding lesioned motoneurons stem from terminals that have lost contact with the injured cells.

### 4.2.5 Confocal microscopy

Confocal microscopy is today a routine method for acquisition of images of immunostained tissue. The method offers very high resolution and detection of signal that can be hard to perceive with conventional light microscopy. Important aspects of confocal microscopy include detection levels and colocalization accuracy.

First, it is almost always possible to get a signal with a confocal microscope if one uses enough laser power and electronic amplification in the detector. In this thesis, all images have therefore been acquired in parallel with negative controls – identical tissue that has been subjected to the exact same experimental conditions, except exposure to the primary antibody. The negative controls were scanned with the exact same settings as those of the experimental specimens and did not result in any signal.

Second, we use confocal images to measure immunoreactivity. Just as with ISH, there can be large variations in levels of immunoreactivity between specimens and for that reason we only compare immunoreactivity in one part of a spinal cord section with the immunoreactivity detected with the exact same microscope settings in a different part of the same spinal cord section. Just as ISH signal should not be seen as an absolute quantification of mRNA, measurements of immunoreactivity should not be

interpreted as absolute quantifications of the protein content of a tissue. As with all semiquantitative measurements, saturation of signal or weak signal can distort the measurements.

Finally, we use confocal microscopy to study the relation between immunoreactivity for different proteins. Confocal microscopy offers several pitfalls in colocalization studies due to optical errors and improper settings. We have used small pinholes in our colocalization studies and performed 3D-stacks where possible. However, confocal microscopy has its limits in imaging of very fine structures, which we have taken into account in our conclusions.

# 5 RESULTS AND DISCUSSION

## 5.1 ADHESION MOLECULES

The initial papers (I-IV) upon which this thesis is based deals with the expression of cell-cell adhesion molecules in axotomized spinal motoneurons.

### 5.1.1 Nectins

Nectin-1 and -3 were selected for analysis because of their demonstrated roles in synapse formation (Mizoguchi et al., 2002). No distinct accumulation of signal for nectin-1 mRNA could be seen over uninjured motoneurons, but such a signal was observable over injured motoneurons (paper I). Other investigators have described a low expression of nectin-1 in spinal motoneurons (Haarr et al., 2001), which was possibly below the detection level of our radioactive ISH method. ISH signal for mRNA encoding nectin-3 displayed a more complex expression pattern, with signal only in a subpopulation of cells in uninjured animals. These cells were positive for VAChT mRNA and therefore interpreted as motoneurons (Roghani et al., 1994). After axotomy, signal for nectin-3 mRNA appeared over most motoneurons (paper I).

Distribution of nectin proteins was examined by IHC. Immunoreactivity for both nectin-1 and -3 was detected in the ventral horn of the spinal cord, but for none of the molecules did the signal colocalize with that for synaptophysin. The extrasynaptic immunoreactivity colocalized with signal for dendritic and axonal markers as well as signal for the astrocyte marker GFAP (Zelano, unpublished observations).

In addition to providing synaptic adhesion, nectins guide axons during development (Okabe et al., 2004b). We therefore examined immunoreactivity for nectins in lesioned sciatic nerves and detected an increase in signal for nectin-3. The signal for nectin-3 colocalized with signal for the axonal marker neurofilament as well as the Schwann cell marker S100 (paper I). ISH revealed an increased signal for nectin-3 mRNA over the stumps of lesioned nerves (paper I), in line with an increased expression of the molecule in Schwann cells.

### 5.1.2 Nectin-like molecules in the peripheral nerve

Necl-1, -2, and -5 are able to interact with nectin-1 or -3 (see introduction). Given our findings that lesioned motoneurons increase their expression of nectin-1 and -3, we investigated the expression pattern of necls that could be potential binding partners for nectin-1 or -3 in motoneurons and lesioned sciatic nerves.

Signal for necl-1 (SynCAM3) mRNA was detected in motoneurons and the expression persisted without detectable change after SNT (paper II). Signal for necl-1 mRNA was also present in the sciatic nerve in a pattern suggesting Schwann cell expression (paper III). Following axotomy, signal for necl-1 mRNA was diminished in the stump of the severed nerve, where Schwann cells had begun to proliferate. Our results in unlesioned nerves were consistent with those of other investigators (Maurel et al., 2007; Spiegel et al., 2007). Signal for necl-2 (SynCAM1) mRNA decreased over lesioned motoneurons and was not detected over control or lesioned sciatic nerves (papers II and III). Signal for necl-3 (SynCAM2) remained unchanged over motoneurons after axotomy (see below in the SynCAM1 and -2-section).

Necl-4 (SynCAM4) interacts with axonal necl-1 to mediate myelination (Maurel et al., 2007; Spiegel et al., 2007). Signal for necl-4 mRNA could be detected in uninjured sciatic nerves, but increased markedly in the stump of severed nerves, in the area where signal for necl-1 mRNA was decreased. In agreement with previous reports (Maurel et al., 2007; Spiegel et al., 2007), we detected high signal for necl-4 mRNA in postnatal nerves (paper III).

Necl-5 interacts with nectin-3 to regulate cell migration and growth (Fujito et al., 2005). We detected an increased signal for necl-5 mRNA in the stumps of severed nerves and over lesioned motoneurons (paper III).

On the protein level, we used an antibody raised against necl-2, but with affinity also for necl-1 and -3. Necl immunoreactivity was detected in the spinal cord, where it localized to synaptophysin-positive structures (paper II, IV). Necl immunoreactivity was also seen in the peripheral nerve, where it localized to structures positive for either the axonal marker neurofilament or the Schwann cell marker S100, both in control and lesioned nerves, but not to structures positive for the macrophage marker ED1 (paper III).

### 5.1.3 Possible roles of nectins and necl-1, -4, and -5.

Our findings on the expression of nectins and nectin-binding necls are summarized in figure 1. As demonstrated, lesioned motoneurons mobilize several members of the nectin and necl families of adhesion molecules. In addition, binding partners for motoneuron nectins or necls are expressed in the peripheral nerve.

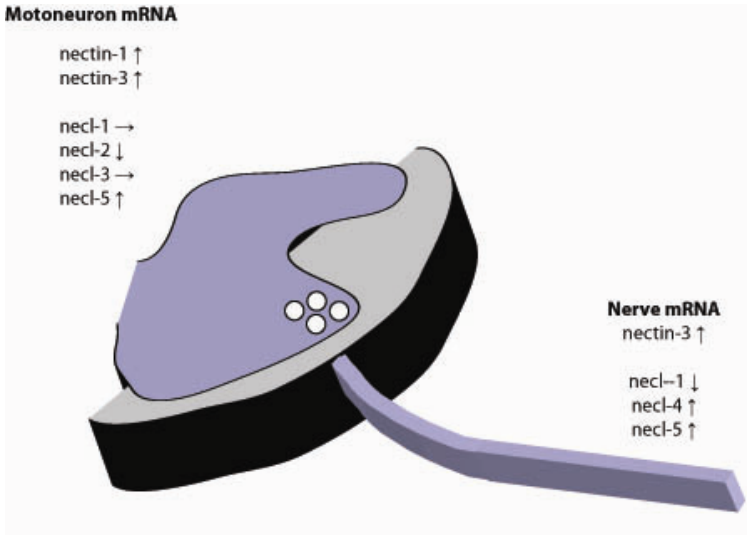


**Figure 1. Alterations in nectin and necl mRNAs after SNT.**

First, we were not able to demonstrate nectin protein localized to synapses on motoneuron cell bodies. Thus, our findings do not indicate a role for nectins in stable synapses on motoneurons and disruption of nectin adhesion is therefore not a likely mechanism for synaptic stripping. Nectins have been suggested to mediate initial adhesion in the formation of synapses and then recruit N-cadherin. Since immunoreactivity for N-cadherin colocalized with signal for synaptic markers on the surface of motoneurons (see below), we cannot exclude that the increased expression of

nectins in lesioned motoneurons is important for the return of synapses following regeneration. However, since this would be difficult to asses and since our main focus was the elimination of synapses, we did not pursue this line of enquiry further.

Immunoreactivity for both nectin-1 and -3 colocalized with signal for the astrocyte marker GFAP and signal for nectin-3 also colocalized with signal for dendritic and axonal markers. Astrocytes interact closely with lesioned motoneurons (Reisert et al., 1984), and this expression pattern indicates that nectins are possible mediators of interactions between astrocytes and motoneurons.

Our findings also point to a role for nectins and necls in peripheral regenerative events. Nectin-3 guides axons during development (Okabe et al., 2004b) and immunoreactivity for the molecule was increased in the injured sciatic nerve (paper I). The immunoreactivity localized to axons, indicating that the increased nectin-3 mRNA levels in motoneurons results in axonal protein. Nectin-3 is able to mediate homophilic adhesion and since nectin-3 immunoreactivity was also localized to S100-positive cells, and since ISH signal for nectin-3 mRNA increased in the stump of the lesioned nerve, it is possible that axonal nectin-3 interacts with Schwann cell nectin-3 during regeneration.

In addition to homophilic interaction, nectin-3 also mediates heterophilic adhesion with nectin-1 and necl-1, -2, and -5. Nectin-1 mRNA or protein was not detected in the sciatic nerve, nor was necl-2 mRNA (papers I and III). Necl-5 has been shown to mediate reduced cell proliferation and movement upon interaction with nectin-3 (Fujito et al., 2005). ISH signal for necl-5 mRNA increased both in lesioned motoneurons and in the lesioned sciatic nerve, making any resulting protein well positioned for interaction with nectin-3. The cell type expressing necl-5 in the lesioned nerve remains to be determined.

Axonal necl-1 mediates myelination by interacting with Schwann cell necl-4 (Maurel et al., 2007; Spiegel et al., 2007). Our ISH indicates that SNT causes Schwann cells to resume the phenotype displayed during postnatal myelination, with a high expression of necl-4 mRNA (paper III). Meanwhile, motoneurons maintain high levels of necl-1 mRNA expression (paper II). This makes it plausible that increased Schwann cell expression of necl-4 results in interactions with necl-1 on regenerating axons, an interaction that is required for myelination. The role of a downregulation of necl-1 expression in Schwann cells in lesioned nerves is less clear. It constitutes a return to the developmental phenotype of the cell, but whether decreased necl-1 adhesion disinhibits Schwann cell or axonal processes in the lesioned nerve remains to be determined.

### 5.1.4 N-cadherin and NCAM

N-cadherin and NCAM are adhesion molecules with roles in synaptic adhesion as well as axon-Schwann cell interactions. We detected signal for N-cadherin and NCAM mRNA in spinal motoneurons, in agreement with the findings of other investigators (Monks and Watson, 2001; Prieto et al., 1989). Signal for N-cadherin mRNA remained unchanged throughout the first two weeks after SNT, whereas signal for NCAM mRNA increased (paper I).

IHC revealed N-cadherin immunoreactivity on the surface of spinal motoneurons, where it colocalized with immunoreactivity for synaptophysin (paper I). One week after SNT, this staining was decreased. N-cadherin is a well known synaptic adhesion molecule in several regions in the CNS (Benson and Tanaka, 1998; Huntley and

Benson, 1999; Jontes et al., 2004), and our findings demonstrate a localization of the protein that is well in line with a role in synapses on sciatic motoneurons. The loss of signal on sciatic motoneurons may reflect a role in synaptic adhesion, since it occurs at a time when synapses are lost from lesioned motoneurons. Our findings fit well with those of Eleore and co-workers who investigated facial motoneurons after axotomy and detected a loss of pan-cadherin immunoreactivity on the surface of these cells (Eleore et al., 2005).

Since N-cadherin localized to synapses on motoneurons, disruption of N-cadherin adhesion could contribute to the loss of synapses from lesioned motoneurons, but the mechanisms underlying the decreased N-cadherin expression remain obscure. The decrease does not seem to be caused by decreased transcription, since signal for N-cadherin mRNA was unchanged after SNT (paper I). Other possible mechanisms include a selective transport of N-cadherin protein to the regenerating axon and/or perhaps cleavage of synaptic N-cadherin by metalloproteinases (Monea et al., 2006; Reiss et al., 2005).

### 5.1.5 SynCAMs 1 and -2

SynCAM1 (necl-2) is an adhesion molecule with synapse-inducing properties (Biederer et al., 2002). In paper II, we investigated the expression of SynCAM1 in spinal motoneurons, and found ISH signal for SynCAM1 mRNA over unlesioned neurons, a finding which has been confirmed by other investigators (Thomas et al., 2008). Following SNT, signal for SynCAM1 mRNA was downregulated to <30% of the contralateral signal three days after injury. At this time point, no loss of synaptophysin immunoreactivity could be seen in the lesioned motoneuron pool (paper II).

These findings prompted a more thorough analysis of SynCAM1 expression in relation to synapse remodeling of lesioned motoneurons. Our initial hypothesis was that expression of synaptic adhesion molecules was involved in controlling the synaptic afferens to motoneurons. We therefore sought lesion models that would allow us to study SynCAM1 expression in relation not only to the removal of synapses but also to the return of synapses to motoneurons. SNT is a good model for acute lesions, since it undoubtedly results in axotomy. However, SNT is followed by regeneration which is highly variable with regard both to the number of regenerating axons and the sorting of axons, in contrast to the regeneration seen after sciatic nerve crush (SNC), a lesion model in which the supporting tissues of the nerve are left intact and severed axons thereby given the opportunity to regenerate in the anatomically correct location (Lago and Navarro, 2006). In paper IV, we used SNT and SNC to induce synapse elimination and determined that both lesion models resulted in similar synapse elimination but different degrees of synapse restoration after regeneration. The use of SNC and SNT also gave us the opportunity to study whether factors present in the distal stump of the nerve could stimulate SynCAM1 expression.

The signal for SynCAM1 mRNA was decreased over motoneurons after both SNT and SNC three days after lesion. Following regeneration after SNT, immunoreactivity for synaptophysin in the sciatic motoneuron pool displayed a significant correlation to ISH signal for SynCAM1 mRNA. Following regeneration after SNC, synaptophysin levels had returned to normal levels, as had the ISH signal for SynCAM1 mRNA.

SynCAM2 (necl-3) also has synapse-inducing properties and mediates synaptic adhesion *in vivo* (Fogel et al., 2007). We detected ISH signal for SynCAM2 mRNA over unlesioned motoneurons but could not detect any significant changes in signal strength after SNT or SNC (paper IV).

On the protein level, we investigated SynCAM/necl expression using an antibody raised against SynCAM1, but with affinity for SynCAM1-3. Western blot demonstrated expression of all three molecules in the ventral spinal cord (paper IV). SynCAM immunoreactivity was colocalized with synaptophysin immunoreactivity on the surface of spinal motoneurons, and SynCAM immunoreactivity decreased on the surface of these cells after axotomy (paper IV).

Taken together, we show that SynCAMs are expressed by spinal motoneurons, present in synapses on these cells, and that axotomy induces a rapid decrease in SynCAM1 mRNA expression in motoneurons. *In vitro*, SynCAM1 expression drives synapse formation (Biederer et al., 2002) and it is therefore possible that decreased expression of the molecule in lesioned motoneurons is one component in the machinery leading to synapse elimination. The initial decrease in signal for SynCAM1 mRNA does not seem to be influenced by the quality of the contact with the distal nerve stump.

### 5.1.6 Neuroligins

NLGs are postsynaptic adhesion molecules with synapse-inducing properties (Chih et al., 2005). *In vivo*, the molecules seem to affect not so much the formation of synapses, as their function and maturation (Südhof, 2008; Varoqueaux et al., 2006). We detected expression of NLG2 and NLG3 mRNA in spinal motoneurons, but no accumulation of signal was seen for NLG1 mRNA. Relatively lower NLG1 mRNA expression compared to that of NLG2 and NLG3 has been described in other parts of the adult nervous system, specifically the brainstem, thalamus, and hypothalamus (Varoqueaux et al., 2006).

In paper II, we detected a decrease in ISH signal for NLG2 and NLG3 mRNA over motoneurons after SNT. The decrease was detectable already at three days after SNT, and thereby preceded reduction of synaptophysin immunoreactivity in the motoneuron pool. We also investigated the mRNA expression levels of PSD-95, a scaffolding protein for NLGs in excitatory synapses, and detected a downregulation that was more gradual than that seen for the NLGs. This downregulation fits well with a previous report on decreased PSD-95 mRNA in facial motoneurons after axotomy (Che et al., 2000).

Similarly to the case of SynCAM1, we asked whether expression of NLGs could be involved in restoration of synapses and whether factors in the distal nerve stump stimulates NLG expression. In paper IV, we demonstrated expression of NLG proteins in the spinal cord by western blot and studied the long term regulation of NLG expression after SNT and SNC. As expected from paper II, signals for mRNAs encoding NLG2 and NLG3 were decreased two weeks after SNT. Seventy days after SNT, the signal for NLG2 mRNA was 85% of control and signal for NLG3 mRNA had been restored, although the return of synapses was highly variable in this lesion model. The downregulation of NLG2 and NLG3 was not as pronounced in the acute phase (3 and 14 days) after SNC as after SNT at these time points, although the loss of synapses was similar in both lesion models. Seventy days after SNC, signal for NLG3 mRNA was higher than over contralateral motoneurons in two experiments, although only

significantly elevated compared to unlesioned controls if all animals from both experiments were pooled (paper IV). Correlation analysis revealed that after regeneration after SNC, signal for NLG2 mRNA correlated to synaptophysin immunoreactivity. In the SNT model, signal for NLG3 mRNA displayed a negative correlation to immunoreactivity for Vglut2, a marker for excitatory synapses.

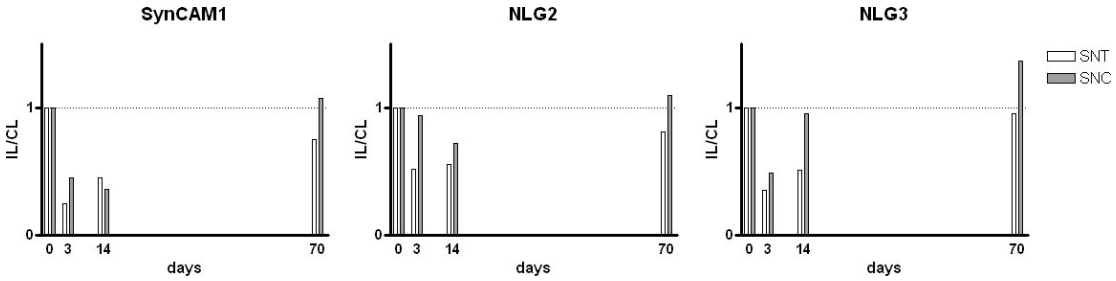


**Figure 2. Comparison of SynCAM1 and NLG mRNA signal levels after SNT and SNC (papers II and IV)**

The findings regarding NLGs do not offer an as straightforward interpretation as those on SynCAM1. Recent studies show that NLG deficient mice display altered synaptic transmission, but a fairly normal number of synapses in the investigated regions (Blundell et al., 2009; Fu and Vicini, 2009; Varoqueaux et al., 2006), which indicates that NLGs may have other roles than determining synapse number. One suggested role is the organisation of synapses by recruitment of receptors and postsynaptic proteins (Fu and Vicini, 2009; Poulopoulos et al., 2009). The pattern of NLG expression in motoneuron regeneration may therefore reflect involvement of NLGs in the maturation of newly formed synapses on motoneurons, regulation of transmission strength, or other synaptic mechanisms.

The expression pattern of NLGs after injury suggests that the expression of these molecules is regulated by other mechanisms than that of SynCAM1. Following SNC, the downregulation of NLGs is less pronounced than that seen after SNT. One of the most prominent differences between these lesion models is the trophic support delivered to the motoneurons from the distal stump of the nerve after SNC. The altered NLG expression after SNC compared to SNT may reflect support from the distal stump by for instance neurotrophins, which can regulate the expression of cell-cell adhesion molecules (Thornton et al., 2008). In a recent study, neurotrophin administration in the distal stump of the severed nerve was shown to reduce the synaptic stripping of motoneurons (Davis-Lopez de Carrizosa et al., 2009).

Importantly, presence of NLG protein in synapses on spinal motoneurons remains to be demonstrated. The NLG mRNA detected in our studies could theoretically be translated to protein that is involved in other adhesive events than synaptic adhesion. However, in addition to the demonstrated mRNA expression in motoneurons, our western blot detected NLG protein in the spinal cord (paper IV). Furthermore, NLGs are present in other CNS synapses in the rat (Song et al., 1999; Varoqueaux et al., 2004), NLG deficiency causes altered brainstem synaptic transmission (Varoqueaux et al., 2006), and NLG proteins are not present in skeletal muscle (Budreck and Scheiffele, 2007; Varoqueaux et al., 2004), making a role in spinal cord synapses likely for any NLG protein resulting from motoneuron mRNA.

## 5.2 COMPLEMENT C3

In 2007 Stevens and co-workers demonstrated that the complement cascade is involved in synapse elimination in the visual system (Stevens et al., 2007). According to their findings, complements C1q and C3 tag synapses, which are then removed. We asked whether the complement cascade could be involved in synaptic stripping of axotomized adult motoneurons (paper V).

We first investigated the expression of parts of the complement cascade after SNT and our findings agreed with previous reports (Svensson and Aldskogius, 1992; Svensson et al., 1995). Signal for mRNAs encoding C1qA-C displayed a clear upregulation in the sciatic motoneuron pool with an expression pattern indicating glial rather than neuronal expression. Immunoreactivity for C1q also seemed to localize to the surface of lesioned motoneurons. Importantly, peripheral nerve lesions are also followed by complement expression at the lesion site, where complement participates in the Wallerian degeneration (Dailey et al., 1998; Ohlsson et al., 2003; Ramaglia et al., 2007). Also in our hands, immunoreactivity for C1q and C3 was detected in the transected sciatic nerve.

We next investigated synapse elimination after injury and found that synaptophysin immunoreactivity was decreased to a larger extent in WT than in $C3^{-/-}$ mice. Similarly, immunoreactivity for Viaat, a marker for inhibitory synapses, displayed a larger decrease in WT animals, whereas the immunoreactivity for the excitatory synapse marker Vglut2 displayed similar levels in WT and $C3^{-/-}$ mice. These findings were confirmed by EM, where lesioned motoneurons in $C3^{-/-}$ mice displayed a larger number of synapses on their cell bodies than lesioned motoneurons in WT mice. We also investigated C1q and C3 immunoreactivity in relation to stainings for synaptic markers on the surface of motoneurons. Some complement-immunoreactive puncta were localized in close proximity to immunoreactivity for synaptic markers, but many were not.

In summary, motoneurons retain more synapses after lesion in the absence of C3. A suggested role for the elimination of synapses from motoneurons is that decreased signalling allows the motoneuron to divert resources to regeneration rather than transmission (see introduction). Therefore, we wondered whether the retained synapses would hamper regeneration. We first performed ISH for GAP-43, a well known regeneration-associated gene, and were surprised to find that the normal upregulation of GAP-43 which occurs in lesioned motoneurons was two-fold increased in $C3^{-/-}$ animals.

We next performed behavioural experiments on $C3^{-/-}$ and WT animals. We performed SNC rather than SNT, as the former lesion results in more standardized regeneration. We evaluated toe-spread, grid walk, and grip strength. We did not detect any difference in the first two parameters. Regarding grip strength, WT and $C3^{-/-}$ mice regained insufficient grip at the same time, but full recovery in $C3^{-/-}$ mice preceded full recovery in WT mice by 4-6 days. At the time of this behavioural difference, we detected higher signals for synaptophysin and Viaat in the spinal cords of $C3^{-/-}$ mice, while the signal for Vglut2 was similar in both strains. No differences were detected regarding inflammation of the lesioned nerve, assessed by CD68 immunoreactivity, and reinnervation of target muscle, assessed by synaptophysin immunoreactivity of motor endplates. In our hands, reduced grip strength returned simultaneously in both mouse strains and, as described above, muscle reinnervation seemed to be fairly similar – altogether we could not detect any altered axonal regeneration speed in $C3^{-/-}$ mice,

which has been described in rats depleted of serum C3 (Dailey et al., 1998). This may be due to species differences, the temporal resolution of our behavioural experiment, or that complete lack of C3 results in a different response than that seen after peripheral depletion of C3.

In summary, the only histological difference detected between mutant and WT mice at the time of the behavioural difference was a stronger immunoreactivity for synaptic markers in the spinal cords of $C3^{-/-}$ animals. Conceivably, preserved synapses may explain the behavioural phenotype, since motoneurons that are better connected to their original networks could be more ready to resume normal function upon target reinnervation.

Importantly, our CD68-findings do not mean that the nerve inflammation is normal in $C3^{-/-}$ mice. It most likely is not, since a vast literature supports a role for complement in recruitment of macrophages to lesioned nerves (Dailey et al., 1998; Li et al., 2007; Ramaglia et al., 2007). However, complement has only been shown to be required for the initial rapid recruitment of macrophages into lesioned nerves. In for instance $C6^{-/-}$ rats, Wallerian degeneration is initially delayed, but axonal and myelin degradation is normal after seven days also in complement deficient animals (Ramaglia et al., 2007).

Our data does not contradict tagging of synapses on lesioned motoneurons, as occurs in the visual system (Stevens et al., 2007). In favour of the tagging hypothesis, complement is present in close relation to lesioned motoneurons and deficiency of complement C3 leads to reduced synaptic stripping. However, tagging is not the only possible explanation for the preservation of synapses in $C3^{-/-}$ animals. This phenomenon may also reflect other roles of complement, for instance chemoattraction of inflammatory cells or glia in the spinal cord or in the lesioned nerve, effects on the gene expression of such cells, or direct effects on lesioned neurons (Woodruff et al., 2008).

Nonetheless, our findings have several implications. First, C3 deficiency preserves synapses on motoneurons, indicating that the complement system may be a target for therapeutic interventions attempting to preserve synaptic networks in the spinal cord. Furthermore, it seems that loss of synapses is not a prerequisite for motoneuron regeneration.

## 5.3 SYNCAM1 AND NEUROLIGINS IN MICE WITH ALTERED SYNAPSE ELIMINATION

In paper V, we describe decreased synaptic stripping in mice lacking complement C3. This contrasts to the increased synaptic stripping that occurs in mice lacking functional MHC class I molecules (Oliveira et al., 2004). We asked whether the expression of synaptic adhesion molecules in motoneurons reflects the altered synaptic stripping in the MHC class I-deficient and $C3^{-/-}$ mice. If the expression of synaptic adhesion molecules follows the number of synapses in these models, it would indicate that expression of synaptic adhesion molecules is either a consequence of synapse removal or a final common pathway in the process of synapse elimination.

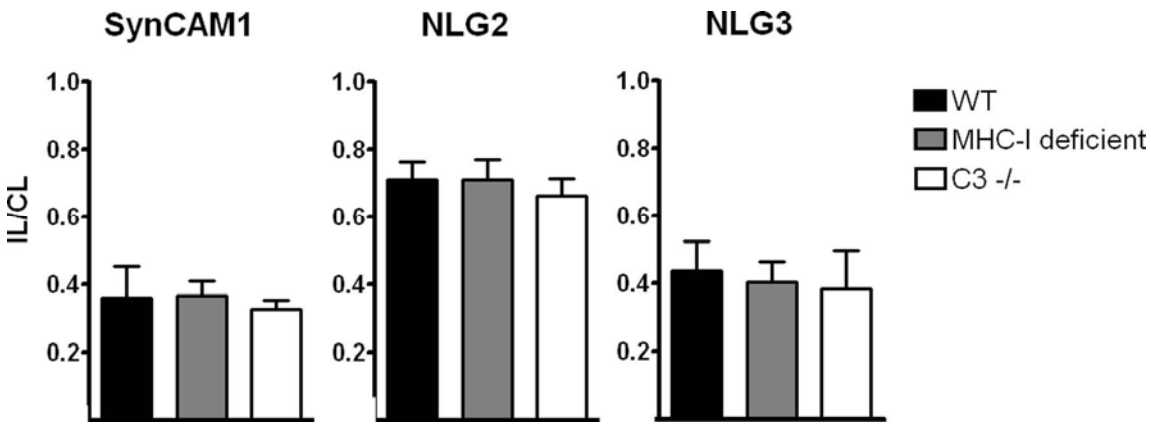


**Figure 3. ISH for SynCAM1, NLG2, and NLG3 in motoneurons 7 days after SNT. n=6/group. Error bars indicate SD.**

If the expression of synaptic adhesion molecules does not reflect the altered synaptic stripping, it would indicate that altered expression of such molecules is one aspect of the neuronal response to axotomy, but that the removal of synapses depends on other independent processes as well.

We performed ISH for SynCAM1, NLG2, and NLG3 in $C3^{-/-}$ and MHC class I-deficient animals seven days after SNT. We did not detect any difference in expression of the investigated molecules in either strain compared to WT animals (Zelano et al, unpublished observations). Thus, it seems that the downregulation of SynCAM1, NLG2, and NLG3 is a neuronal response that occurs regardless of the effects mediated by C3 or MHC-I molecules and that the altered synapse remodeling in the investigated mouse strains is not due to differences in expression of SynCAM1, NLG2, or NLG3.

## 5.4 MAIN CONCLUSIONS

This thesis provides evidence that axotomy of spinal motoneurons results in altered expression of a number of cell-cell adhesion molecules that have previously not been investigated in the context of motoneuron regeneration. The expression patterns and developmental roles of these molecules indicate involvement in a wide range of processes. Specifically, we have shown that:

- Spinal motoneurons express N-cadherin, NCAM, SynCAM1/necl-2, SynCAM2/necl-3, SynCAM3/necl-1, NLG2, and NLG3 and a subpopulation of spinal motoneurons express nectin-3.

- Lesioned motoneurons increase their expression of nectin-1 and -3 and necl-5 and the protein localization of nectins and certain necls suggests involvement in neuron-glia interactions both in the spinal cord and the peripheral nerve.

- Binding partners of motoneuron nectins and necls are expressed in lesioned nerves, indicating that these families of adhesion molecules may be involved in axon regeneration, including axon-glia interactions. Regarding necl-1 and necl-4, the expression pattern of the molecules closely resembles that seen during postnatal myelination.

- Expression of SynCAM1, NLG2, and NLG3 is rapidly decreased in lesioned motoneurons and returns during regeneration. In the case of SynCAM1, the expression is restored in close relation to the return of synapses. Synaptic stripping of motoneurons occurs regardless of the quality of the contact with the distal stump of the nerve and good contact enhances motoneuron NLG expression but does not influence SynCAM1 expression. The expression of SynCAM1 and NLGs is acutely reduced to a similar degree in mice with altered synaptic stripping and wild type mice.

We conclude that axotomized spinal motoneurons decrease their expression of SynCAM1 and NLG2 and -3, and that the expression of these molecules increases prior to or in close relation to the return of synapses to motoneurons. However, the relation between restored expression of SynCAM1 and NLGs and the return of synapses is not clear, nor does the magnitude of the initial synaptic stripping seem to solely depend on the magnitude of the decreased expression of these molecules.

This thesis also provides evidence for a role for complement C3 in synaptic stripping. Specifically, we have shown that:

- Complement $C3^{-/-}$ mice display reduced removal of synapses from lesioned motoneurons, an increased expression of GAP-43 by these cells, and a more rapid restoration of motor function. In WT mice, expression of complement C3 and C1q is increased within the spinal cord, but does not solely localize to synapses.

We conclude that complement C3 is required for normal synaptic stripping of axotomized motoneurons.

# 6 GENERAL DISCUSSION AND FUTURE DIRECTIONS

We demonstrate that several recently discovered cell-cell adhesion molecules are expressed by spinal motoneurons and that the motoneuron response to axotomy involves more complex alterations in the expression of cell-cell adhesion molecules than has previously been known. Furthermore, we study the possible involvement of complement, the most recently described mediators in CNS synapse elimination, in synaptic stripping of adult motoneurons.

Specifically, we show that nectins are mobilized by motoneurons and glia after axotomy. Since regeneration after peripheral nerve lesions is considered successful when compared to the regeneration seen after axonal injuries within the CNS, it would be interesting to see whether for instance nectin-3 and necl-5 are expressed also after CNS lesions, and the effect of modulating their expression after both central and peripheral injuries. We also describe changes in necl-1 and -4 expression levels that resemble those seen during postnatal myelination, indicating involvement of the molecules in postinjury remyelination. Recently, lack of necl-1 has been shown to delay myelination within the CNS (Park et al., 2008) and a natural next step for the research on necl-1 and -4 could be to investigate their roles in adult demyelinating diseases, such as multiple sclerosis.

Regarding the synaptic adhesion molecules SynCAM1 and NLGs, our findings fit well with synaptic roles for these molecules in motoneurons and with the notion of a switch, where injured motoneurons reduce the expression of their transmission machinery and increase their regeneration capacity. Our findings show that a downregulation of SynCAM1 and NLGs does occur after axotomy, but also indicate that the motoneuron levels of these molecules are not the sole determinants of the magnitude of the loss of synapses. Most likely, future studies will reveal more complex roles for NLGs and SynCAMs in motoneurons than merely determining synapse number. The possibility remains that downregulation of synaptic adhesion molecules such as SynCAM1 may be required, but not sufficient, for synaptic stripping. Similarly, restored expression of SynCAM1 and NLGs may be required for return of synapses to motoneurons after regeneration. In addition to further analysis of NLGs and SynCAMs, future studies on synaptic adhesion molecules in motoneurons could include other even more recently discovered molecules with synapse-inducing properties (Brose, 2009).

Many questions remain regarding the preservation of synapses in $C3^{-/-}$ mice. Immuno-EM can determine whether complement does indeed localize to synapses undergoing elimination. Neurophysiology on nerve can determine whether subtle differences exist between $C3^{-/-}$ and WT mice in remyelination or axonal regeneration, and motor evoked potentials might be able to determine whether a preserved circuitry within the spinal cord can really result in increased motor strength. Efforts should also be directed at determining the mechanism of complement action – which receptors are important for the effect? Does elimination of other complement factors result in the same response?

It remains a matter of controversy whether the loss of synapses from lesioned motoneurons is the result of glial actions, neuronal processes, or both. Several ways to modulate synaptic stripping are now known, such as abolished MHC class I or C3 expression and administration of neurotrophins (Davis-Lopez de Carrizosa et al., 2009).

These diverse findings indicate that the loss of synapses may not be the result of actions by a single cell type or mechanism, but perhaps reflects the collective impact of several processes on the lesioned motoneuron.

In a clinical perspective, an equally relevant question to what makes synapses disappear is what can make them remain. In this respect, this thesis points to two possible targets in future therapeutic strategies. First, the motoneuron expression of synaptic adhesion molecules is rapidly reduced after axotomy and genetic or pharmacological ways to preserve the expression of these molecules after lesion should be sought to see whether that can halt the synapse elimination. Most likely, a time frame will exist for such an intervention, given the rapid alterations of the motoneuron expression levels of these molecules after lesion. Loss of synapses is an early feature of several disorders in the nervous system (Schutz, 2005; Selkoe, 2002; Zang da et al., 2005; Zhu et al., 2003). Whether this synapse elimination is also preceded by reduced expression of synaptic adhesion molecules is an exciting area for future investigations. In addition to a therapeutic potential, more information on the expression pattern of synaptic adhesion molecules might contribute to improved diagnostic tools and disease criteria.

Second, the inflammatory response after lesion, specifically complement C3, also seems to influence the removal of synapses. The view on complement is currently undergoing a paradigm shift due to the fact that several new roles for the molecules, unrelated to host defence, have been demonstrated within the CNS. Complement receptors are expressed on all cell types within the CNS, and complement is required for several seemingly beneficial processes, such as neurogenesis and increased glutamate uptake in microglia (Persson et al., 2009; Rahpeymai et al., 2006). However, complement also has negative effects. For instance, treatment with C5a-receptor-antagonist increases the survival of motoneurons in an ALS-model (Woodruff et al., 2008). Complement also participates in processes such as synapse elimination (Stevens et al., 2007), which can be regarded as physiological or pathological, depending on the context. Our findings demonstrate that elimination of C3 not only preserves synapses after motoneuron injury, but also results in an improved clinical outcome. This indicates that the inflammatory response is a possible target for future therapeutic interventions attempting to preserve synaptic circuits. Given the many roles of complement within the CNS, further dissection of the pathway leading to synapse elimination might allow for more precise targeting than elimination of factor C3 in future therapeutic interventions.

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