Elimination of synapses from injured motoneurons – a model for study of synaptic plasticity in the adult central nervous system

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There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after”

**J.R.R. Tolkien**, The Lord of the Rings

*For every problem, there is one solution, which is simple, neat and wrong.*

**Henry Louis Mencken** (1880-1956)

*Result! Why, man, I have gotten a lot of results. I know several thousand things that won’t work.*

**Thomas Edison**
ABSTRACT

Synapses are the contacts between nerve cells or between nerve and muscle cells. The integrity of these synapses is crucial for proper function. Several neurodegenerative diseases such as Alzheimer’s disease, multiple sclerosis and neurotrauma involve synaptic pathology. In this thesis we have used the nerve lesion models sciatic nerve transection (SNT) or sciatic nerve crush (SNC) to enable the study of events leading to synaptic stripping and subsequent reformation after lesion.

The aim of this thesis was to investigate the role of specific factors that mediate the response of the spinal cord to peripheral axotomy, particular emphasis was placed upon molecules and cell populations that could have an influence upon the synaptic stripping of lesioned motoneurons. Following axotomy glial cells – i.e. microglia and astrocytes surrounding the lesioned motoneurons – are activated and proliferate and interact intimately with lesioned neurons. Furthermore, these glial cells express and secrete complement factors that supposedly ‘tag’ synapses destined to be removed as suggested by Stevens et al. 2007. Simultaneously, motoneurons down-regulate the expression of several adhesion molecules important for the maintenance of structural integrity and this is followed by the removal of synapses.

In paper I and II, we studied the adhesion molecules SynCAM1, neuroligin 2 and -3 and Netrin G-2 ligand (NGL-2). In vitro these adhesion molecules can all induce synapse formation. They are all expressed by motoneurons and down-regulated after axotomy before synaptic stripping occurs. SynCAM1 expression correlates to loss and return of synapses in the SNT model. The expression levels of the neuroligins decreased to a smaller extent after SNC than SNT, suggesting that the contact with the distal nerve stump is important for the expression levels of the neuroligins and did not display as a clear correlation with synapse numbers as SynCAM1. NGL-2 displayed a lower general expression by motoneurons and was down-regulated to a similar extent both in the SNT and SNC model.

In paper III, we investigated the role of complement components C1q and C3 in the removal of synapses from axotomized motoneurons. In WT mice both C1q and C3 was clearly up-regulated after lesion. C1q−/− mice displayed the same degree of synaptic stripping as WT mice. In contrast, C3−/− mice displayed a hampered stripping response following axotomy that was associated with a preferential loss of inhibitory synapses and increased expression of the regenerative associated protein GAP-43. These effects were accompanied by faster functional recovery. We did not however, see any obvious signs of hampered inflammation at site of lesion. Complement IR was seen in close interaction with the lesioned motoneurons and its dendritic tree. Yet, we did not observe any clear evidence for a ‘tagging’ process as suggested by previous investigators. In paper IV, we compared C3−/− and MHC class Ia deficient mice; two strains exhibiting contrasting responses to axotomy. The C3−/− mice exhibit a hampered stripping process compared to WT mice and MHC class Ia deficient mice have an augmented stripping response compared to WT mice. We asked whether variation in the expression of synaptic adhesion molecules previously studied in motoneurons (SynCAM1, neuroligin -2 and -3, and Netrin g-2 ligand) or changes in activation of microglia and astrocytes reflected the altered synaptic stripping that is seen in these mouse strains. We concluded that neither glia activation nor the down-regulation of synaptic adhesion molecules were correlated to variation in synaptic stripping observed in the two strains studied In paper IV, we examined the effects exerted by astrocytes on the stripping event by the usage of GFAP−/−VIM− mice. We observed a marginally affected stripping response in these mice compared to WT mice and slower functional recovery. The delayed functional recovery was however, most likely due to effects on the lesion site and not in the spinal cord.

To summarize, complement C3 seems to be an important factor in the synaptic stripping event, especially for inhibitory synapses. The effects exerted by complement C3 do not seem to be linked to distorted glial up-regulation or by an affect on the down-regulation of the studied synaptic adhesion molecules. It remains to be unravelled via which pathways and receptors complement exert these effects and whether intervention aimed at the complement system could be used for therapeutic interventions in order to promote synapse preservation in neurodegenerative diseases.
LIST OF PUBLICATIONS

This thesis is based on the following publications and manuscripts.

I. 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 Dec 10;517(5):670-82


*equal contribution


V. **Berg A**, Zelano J, Thams S, Cullheim S. The extent of synaptic stripping of motoneurons after axotomy is not correlated to activation of surrounding glia or down-regulation of postsynaptic adhesion molecules. PLOS One. 2013 March. In press
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
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<tr>
<td>C1q</td>
<td>Complement component 1q</td>
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<td>C3</td>
<td>Complement component 3</td>
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<tr>
<td>ChAT</td>
<td>Choline acetyltransferase</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
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<td>EM</td>
<td>Electron microscopy</td>
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<tr>
<td>GABA</td>
<td>Gamma amino-butryic acid</td>
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<td>GAP-43</td>
<td>Growth-associated protein-43</td>
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<tr>
<td>GDNF</td>
<td>Glial derived neurotrophic factor</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<td>GluR1</td>
<td>Glutamate receptor 1</td>
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<tr>
<td>IGF-1</td>
<td>Insulin growth factor-1</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>IL/CL</td>
<td>Ipsilateral/contralateral</td>
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<tr>
<td>ISH</td>
<td><em>In situ</em> hybridization</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>mEPSCs</td>
<td>Miniature excitatory post synaptic currents</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<td>NGL-2</td>
<td>Netrin G-2 ligand</td>
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<td>NLG</td>
<td>Neuroligin</td>
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<tr>
<td>NMDF</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NT-3 and -4</td>
<td>Neurotrophins-3 and -4</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PSA</td>
<td>Polysialic acid</td>
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<tr>
<td>PSC</td>
<td>Perisynaptic Schwann cell</td>
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<tr>
<td>PSD-95</td>
<td>Post synaptic density protein-95</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>SALM</td>
<td>Synaptic adhesion like molecule</td>
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<tr>
<td>SNC</td>
<td>Sciatic nerve crush</td>
</tr>
<tr>
<td>SNT</td>
<td>Sciatic nerve transection</td>
</tr>
<tr>
<td>SSC</td>
<td>Standard saline citrate</td>
</tr>
<tr>
<td>SynCAM</td>
<td>Synaptic cell adhesion molecule</td>
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<tr>
<td>VGLUT2</td>
<td>Vesicular glutamate transporter 2</td>
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<tr>
<td>VIAAT</td>
<td>Vesicular inhibitory amino acid transporter</td>
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<tr>
<td>VIM</td>
<td>Vimentin</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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## INTRODUCTION

### 2.1 GENERAL INTRODUCTION

#### 2.1.1 Anatomy of motoneurons

Spinal motoneurons are the final conveyors of signals originating in the motor cortex, travelling down the spinal cord in the corticospinal tract, and synapsing on the motoneurons whose axon finally innervates the target muscle. They are the link between the central nervous system (CNS) and the peripheral nervous system (PNS).

The soma and dendrites of motoneurons are primarily located in the grey matter of the dorsal part of the ventral horn in layer VIII and IX according to the nomenclature of Rexed (Rexed, 1954), and are protected from the outer milieu by the blood brain barrier (BBB). A motoneurons extensive dendritic tree receives hundreds of thousands of synaptic inputs (Ornung et al., 1998). The majority of the synaptic inputs to motoneurons are inhibitory and originate from local interneurons, descending motor tracts and primary sensory afferents. This provides the motoneuron with information from higher motor centers, as well as quick reflex circuits protecting the organism from potential hazards.

![Figure 1: The anatomy of sciatic motoneurons.](image)

This picture shows sciatic motoneurons with dorsolateral motoneurons in blue and ventrolateral motoneurons in red. These neurons receive input from higher motor centers via the corticospinal tract (purple). They then extend their axons via the ventral root to form the sciatic nerve, which innervates the hind limb muscles. The axons are surrounded by Schwann cells which myelinate axons enabling fast conduction of electrical impulses. The place of transection is indicated by black line. The sciatic nerve also has sensory fibers (green) projecting to the DRG. The nerve signal is then transmitted via the dorsal root directly to motoneurons and in-directly via interneurons (black). The motoneuron soma, axon and innervated motor fiber make up a complete motor unit.
2.1.2 Synapses

Synapses are the functional sites between neurons where an impulse is transmitted from one nerve cell to another, and was first proposed by Sherrington (M Foster, 1897). The average neuron forms about one thousand synapses and receives up to ten thousand (Ulfhake and Cullheim, 1988) and in the healthy nervous system this is subject to a constant yet varying degree of turnover. In neurotrauma and neurological diseases such as Alzheimer’s disease, multiple sclerosis (MS) and many others (Marques et al., 2006; Selkoe, 2002; Zang da et al., 2005), there is a shift towards removal of synapses in a process called synaptic stripping. Peripheral axotomy is an easy way to induce and study this process as well as the subsequent reoccurrence of synapses on lesioned motoneurons. Simultaneously, there is a massive glial response within the spinal cord and proteins of the complement cascade are up-regulated (Mattsson et al., 1998). Complement proteins have also been implicated in the process of synaptic pruning during development (Stevens et al., 2007). Understanding the basic mechanisms underlying these events can result in new treatments for diseases that are virtually untreatable today.

Principally there are two types of synapses; chemical or electrical. Synapses can be axo-axonic, axo-somatic or axo-dendritic and on rare occasions dendro-dendritic and soma-somatic. A synapse consists of the presynaptic neuron, the terminal contacting the postsynaptic neuron, usually the dendrites or the soma of the post-synaptic neuron and the synaptic cleft. The synaptic terminal contains vesicles filled with either excitatory or inhibitory transmitter molecules. Following an action potential, Ca$^{2+}$ enters the presynaptic terminal causing vesicles to fuse with the presynaptic membrane resulting in transmitter release into the synaptic cleft. The transmitter molecules diffuse to the postsynaptic membrane where they interact with postsynaptic receptors.

In the CNS, glutamate is the principle excitatory neurotransmitter, and GABA and Glycine are the main inhibitory neurotransmitters. Excitatory synapses contact the target neuron via the dendritic spines, while inhibitory synapses contact the soma directly. The result is that the excitatory signal must propagate through the soma to reach the initial axon segment and inhibitory signals, by influencing the somatic milieu, act as a filter.

The synaptic cleft separates the pre- and postsynaptic membranes usually by a distance of about 20-40 nm. Synaptic adhesion molecules hold these two membranes together. Initially, they were thought only to preserve the structural integrity of the synapse. However, over the last decade our understanding of synapses has increased. We now know that some molecules, such as the neuroligins, SynCAM, Netrin G-2 ligand and SALMs have synapse-inducing capabilities (Biederer et al., 2002; Chih et al., 2005; Kim et al., 2006; Ko et al., 2006). Thus, presumably the neuron itself can regulate synapse number and type of synaptic input.

2.1.3 Lesion effects on motoneurons

In the CNS, peripheral axotomy induces several changes in the lesioned motoneuron, as well as in its surrounding environment. This is seen after traumatic injuries as well as in neurological disease. Under these circumstances the motoneuron changes from a transmitting phenotype to a regenerating phenotype whose primary role is the restoration of original function and re-innervation of the target cell. As part of this process the motoneuron changes the expression of several molecules involved in synaptic transmission and nerve regeneration. These include a prominent decrease in the expression of choline acetyltransferase (ChAT), GABA and NMDA-receptor subunits. Contrastingly, the growth-associated protein GAP-43 is vastly up-regulated following axotomy (Davidoff and Schulze, 1988; Linda et al., 1992; Piehl et al., 1991; Piehl et al., 1998; Piehl et al., 1995b).
A few days after axotomy, glial cells- astrocytes and microglia- are activated and interact closely with the lesioned motoneurons (Aldskogius et al., 1999; Blinzinger and Kreutzberg, 1968; Graeber et al., 1988). Synapses are removed from the soma and the dendritic tree of the motoneuron in a process called synaptic stripping (Blinzinger and Kreutzberg, 1968; Chen, 1978; Sumner, 1975), resulting in reduced synaptic input (Eccles et al., 1958). The majority of these synapses do not return following regeneration (Alvarez et al., 2011). This is followed by morphological changes called chromatolysis where dendrites are retracted, the nucleus is decentralized and the disassembly of the Nissl substance occurs.

Much controversy still exists regarding the roles of synaptic stripping. It has been suggested that the removal of synapses protects the neuron from over-excitation and enables the cell to reallocate its resources to better facilitate regeneration (Lindå et al., 2000; Svensson and Aldskogius, 1993). The exact mechanisms for the synaptic stripping process still remain elusive. Several cell types and molecules have been implicated in this process such as microglia, astrocytes and major histocompatibility complex (MHC) class I molecules and more recently complement C1q and C3 (Blinzinger and Kreutzberg, 1968; Oliveira et al., 2004b; Reisert et al., 1984; Svensson and Aldskogius, 1993; Svensson et al., 1993).

### 2.1.4 Lesion effects on nerve, regeneration and neuromuscular innervation

Following axotomy the distal part of the axon undergoes anterograde degeneration, so called Wallerian degeneration (Waller, 1850). In this process, myelin and axonal debris is cleared and recycled by infiltrating macrophages, but also to a lesser extent by Schwann cells (Bigbee et al., 1987; de la Motte and Allt, 1976).

The material ingested by the macrophages provides a mitogenic environment for Schwann cells. The Schwann cells proliferate and guide the outgrowing axons down the endoneurial tubes by providing the basal lamina on one side and the Schwann cell membrane on the other side. Axons continue to grow towards their target, but may branch distally to the site of lesion. However, following regeneration and reinnervation, some axon branches will disappear and other will enlarge and return to original size (Cragg and Thomas, 1964)

A couple of weeks following sciatic nerve lesion there is a period of re-innervation, during which axons travel the distance from the site of lesion to their targets of innervation. At first there is a phase of hyperinnervation where aberrant neuromuscular junctions are formed. In the muscle, the motor axons exhibit little specificity when selecting between different muscle fibers, i.e. fast and slow twitch. However, during the coming weeks there will be a process of synaptic pruning, where Schwann cells actively participate in the retraction of presynaptic motor terminals leading to the original state where one motoneuron innervates one group of muscle fibers (Magill et al., 2007).

### 2.2 ADHESION MOLECULES INVESTIGATED IN THIS THESIS

#### 2.2.1 SynCAM -1 and -2

Synaptic cell adhesion molecules (SynCAM) were first discovered in a synaptic context in 2002 (Biederer et al., 2002). There are four members of the SynCAM family, SynCAM 1-4. Due to structural similarities with the nectins these molecules are sometimes referred to as nectin like molecules (Ikeda et al., 2003), however throughout this these only the name SynCAM will be used.

SynCAM-1 is a single pass transmembrane molecule mediating both homophilic and heterophilic interactions with SynCAM-2 and -3. SynCAM-2 mediates homophilic interactions as well as heterophilic
interactions with SynCAM-1, -3 and -4 (Fogel et al., 2007; Shingai et al., 2003). SynCAM-1 and -2 mRNAs are expressed throughout the developing and adult nervous system (Biederer et al., 2002), and in the spinal cord SynCAM-1 levels are highly dynamic during a phase of synaptogenesis (Thomas et al., 2008). Furthermore, SynCAM-1 mRNA is down-regulated in visual cortex during a period of synaptic plasticity, indicating that SynCAM-1 expression could disinhibit this process (Lyckman et al., 2008).

The expression of SynCAM-1 in non-neuronal cells co-cultured with hippocampal neurons induces fully functional excitatory synapses (Biederer et al., 2002; Sara et al., 2005). Furthermore, expression of only the intracellular domain of SynCAM1 inhibits synapse formation. SynCAM-1 localizes primarily to excitatory synapses but also to a lesser extent to inhibitory synapses. SynCAM-2 localizes to both excitatory and inhibitory synapses (Fogel et al., 2007). Both SynCAM-1 and -2 have the capability to recruit presynaptic proteins (Fogel et al., 2007).

Previously, we have demonstrated that the expression of SynCAM-1 mRNA decreases prior to the loss of synapses after axotomy, suggesting a role for this molecule in synapse maintenance and also that the down-regulation of SynCAM-1 may be a pre-requisite for the occurrence of synaptic stripping (Zelano et al., 2007b).

**2.2.2 Neuroligins**

Neuroligins are a family of neuronal cell surface proteins first discovered as a binding partner for the presynaptic beta-neurexins (Ichchenko et al., 1995; Ichchenko et al., 1996). Four members of the family, NLG 1-4 have been identified in mice and rats, while five genes are known in humans. Expression of NLGs has been found in a wide range of tissues outside of the CNS, but beta-neurexins are not known to be expressed anywhere else.

NLGs belong to the family of CLAMs (cholinesterase like adhesion molecules) with an extracellular cholinesterase-like domain and a highly conserved PDZ recognition peptide in the intracellular domain, which is most likely important for linking them to other synaptic proteins such as PSD-95, S-SCAM and Shank among many others (Irie et al., 1997; Meyer et al., 2004).

Trans-synaptic interaction between neurexins and NLGs induces synapse formation in several different assays. NLG1 primarily localizes to excitatory synapses and triggers de novo formation of presynaptic structures. Interestingly, overexpression of NLG1 not only induces excitatory synapses but also inhibitory synapses (Prange et al., 2004). Contrastingly NLG2 localizes to inhibitory synapses (Song et al., 1999; Varoqueaux et al., 2004), whereas NLG3 localizes to both equally (Budreck and Scheiffele, 2007). Down-regulation of NLGs by RNAi results in a loss of both excitatory and inhibitory synapses, with the largest effect on inhibitory synapses occurring when NLG2 is inhibited. Some controversies exist regarding the consequences of NLG overexpression. Several studies have demonstrated that overexpression of NLGs induces clustering of postsynaptic proteins (Chih et al., 2005) e.g. NLG1, NLG3 and NLG4 induce aggregation of PSD-95 (Graf et al., 2004). However, these results contrast with other findings that demonstrate that overexpression of NLGs does not alter the clustering of postsynaptic proteins like PSD-95, AMPA and NMDA receptor density (Levinson et al., 2005; Prange et al., 2004). Nevertheless it remains clear that clustering of NLGs and its binding partners is critical for synapse development.

Surprisingly, mice lacking functional NLGs exhibit the same number of synapses as wild-type animals, but many of the synapses are dysfunctional. Mice exclusively deficient in NLG1 display the same number of synapses, but a reduced number of EPSCs and mice lacking all NLG subtypes die soon after birth (Conroy et al., 2007; Varoqueaux et al., 2006). These results indicate that neuroligins are not vital for actual synapse numbers but are instead crucial for accurate synapse function. Furthermore, mice deficient in NLG-3 and -4 display autism like behavior (Jamain et al., 2003; Laumonnier et al., 2004).
The expression of NLG-2 and -3 mRNA decreases after sciatic nerve axotomy in the soma of the lesioned motoneurons. This occurs prior to the loss of synapses from these neurons raising the intriguing thought that the down-regulation of NLG may be fundamental for synapse stripping to occur (Zelano et al., 2007b). However, it is not clear how neuroligin levels are restored during the phase of nerve regeneration, nor if and how the expression levels are correlated with synapse restoration.

2.2.3 Netrin G-2 Ligand

There are three known members of the netrin-G ligand family; netrin-G ligand-1 (NGL-1), the first identified member of the family (Lin et al., 2003), followed by the discovery of NGL-2 and -3 (Kim et al., 2006). Another proposed name for the same molecules is leucine-rich repeat containing 4C (LRRC4C), LRR4C/NAG14 and LRR4B/HSM respectively (Lin et al., 2003; Zhang et al., 2005). The NGL proteins are enriched at the postsynaptic density (Sheng, 2006) and interact with their respective presynaptic binding partner, which is for NGL-1 netrin G-1, and for NGL-2 netrin G-2, but not netrin G-1 (Kim et al., 2006). NGL’s mRNA is expressed predominantly in the CNS and exhibits distinct cellular and subcellular distributions e.g. pyramidal neurons in the hippocampus express mRNAs for all NGL family members, but different NGL proteins are differentially distributed to distinct dendritic segments (Nishimura-Akiyoshi et al., 2007).

NGL-1 molecules play a role in the regulation of axonal outgrowth and migration (Lin et al., 2003), while NGL-2 is more involved in synapse formation (Biederer, 2006; Kim et al., 2006). Knockdown of NGL-2 reduces the number and functions of excitatory synapses, while NGL-2 over-expression promotes presynaptic differentiation and induce clustering of molecules like PSD-95, GKAP, Shank and NMDA receptors (Biederer and Scheiffele, 2007).

The netrin-Gs and NGLs have attracted much interest because of their possible involvement in a wide range of diseases. The levels of netrin G-1 and G-2 are reduced in patients suffering from schizophrenia and bipolar disorders implicating a role in disease (Aoki-Suzuki et al., 2005; Eastwood and Harrison, 2008). Furthermore, NGL-2 is also implicated in other diseases such as the suppression of the malignant brain tumor- glioma (Zhang et al., 2005). In glioma biopsies NGL-2 expression is suppressed or absent, conversely expression of NGL-2 suppress tumorigenesis (Wu et al., 2006).

2.3 The complement system in synaptic stripping and motoneuron regeneration

2.3.1 Overview of the complement system

The complement system is part of the innate immune system and provides rapid response to invading pathogens by opsonizing foreign material, attracting immune cells and lysis of foreign cell membranes. Additionally, it clears apoptotic cells and cellular debris (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2002; Zipfel et al., 2007).

The complement system can be activated via four different routes; the classical, the alternative and the lectin pathway, which all converge on complement C3 and the extrinsic pathway which act independently of C3 and C3 convertases. The classical pathway is initiated by the interaction of C1q with either antibodies or any of its other binding partners e.g. pentraxins, polyanions, or apoptotic cells. The interaction with one of its many binding partners induces a conformational change, which leads to the activation of autocatalytic enzymes in C1r. C1r in turns cleave C1s generating active serine proteases. The activated C1s then cleave C4 and C2 which generates the C3 convertase. The lectin pathway is
initiated by the binding of mannose binding lectins to mannose residues on the cell surface. This activates enzymes that cleave C4 to generate the C4 convertase. The alternative pathway is continuously activated by the spontaneous hydrolysis of C3. Thus, the alternative pathway serves to amplify the events already started via the classical or lectin pathway. The extrinsic pathway initiates the formation of complement end products independent of C3 and C5 convertases via components of the blood clotting and fibrinolysis pathways.

The cleavage of C3 will generate activated C3 fragments; C3b and iC3b leading to elimination of target structures by phagocytosis. Moreover, the other cleavage product C3a mediates the recruitment and activation of macrophages (Nordahl et al., 2004; Zhou, 2012). C3b joins with C3 convertase (C4b2a) generating the C5 convertase (C4b2a3b complex), which in turn will be cleaved into C5a and C5b. C5a binds to C5aR on phagocytic cell. Robust activation of C5b results in the activation of the terminal pathway causing cell lysis through the insertion of C5b-C9 (MAC) complex into the cell membranes.

### 2.3.2 Complement C1q and C3 in the nervous system

Traditionally, the CNS was perceived as an immune privileged site. This view has changed lately with increasing evidence showing that the nervous and immune systems interact both in health and disease (Bellander et al., 1996; Mallucci, 2009; Selkoe, 2002; Stevens et al., 2007). Complement proteins can be produced locally in the brain by neurons and glial cells. However, microglia and astrocytes are the main producers of complement both in the healthy as well as in the diseased CNS (Barnum, 1995; Veerhuis et al., 2011).

Recent work showed that complement C3 and the upstream complement component C1q play a critical role for the synaptic refinement during development and retinal degeneration in the visual system. Mice deficient in C1q or C3 exhibit large sustained defects in the synaptic refinement in the dLGN. This effect was proposed to be exerted via the ‘tagging’ of synapses destined to be removed by C1q (Stevens et al., 2007).

Complement proteins are thought to play a role in various neurodegenerative diseases such as Alzheimer’s disease, glaucoma, Huntington’s disease, Parkinson’s disease, multiple sclerosis and also neurotrauma (Nguyen et al., 2002; Stephan et al., 2012; Wyss-Coray and Mucke, 2002). After peripheral nerve lesion complement proteins are up-regulated and play a critical role for the recruitment of macrophages necessary for Wallerian degeneration (Bruck and Friede, 1990; Dailey et al., 1998; Ramaglia et al., 2008). Furthermore, complement proteins are up-regulated in the spinal cord following peripheral nerve lesion (Liu et al., 1995; Mattsson et al., 1998; Svensson and Aldskogius, 1992; Svensson et al., 1995). Many studies point to a prominent role for complement-mediated pathology in the nervous system, but some conflicting studies indicate positive and regulating roles in repair processes (Brennan et al., 2012). Accordingly, we wanted to study the role of complement on synaptic stripping and recruitment of glial cells in a peripheral nerve lesion model.

### 2.4 GLIAL CELLS INVOLVED IN SYNAPTIC STRIPPING AND MOTONEURON REGENERATION

#### 2.4.1 Astrocytes

Astrocytes are the most abundant cell type in the CNS and outnumber neurons by over fivefold (Sofroniew and Vinters, 2010). As with all glial cell types, the general view has been that astrocytes are a supportive component of neural tissue and not actively contributing to synaptic events and plasticity.
In general there are two types of astrocytes, protoplasmic and fibrous. Protoplasmic astrocytes are found in the gray matter and exhibit several stem branches that give rise to multiple finely branching processes. Fibrous astrocytes on the other hand are found in the white matter and instead exhibit many long fiber-like processes (Ramon Y, 1909). Both cell types make extensive contacts with blood vessels and constitute the BBB and regulate cerebral blood flow in response to neuronal activity (Koehler et al., 2009).

In the healthy CNS, processes from one astrocyte can envelope up to 100,000 synapses on many different neurons (Halassa et al., 2007b). Astrocytes extend their processes to synaptic regions and play a critical role in controlling and regulating ion-, pH-, and transmitter homeostasis. Furthermore, astrocytes contribute to transmitter homeostasis by expressing high levels of transporters for neurotransmitters like glutamate, GABA and glycine (Sattler and Rothstein, 2006). Recently, it has been demonstrated that astrocytes also play an active role in synaptic transmission and actually release molecules like glutamate, ATP, GABA in response to neuronal activity. This has given rise to the expression of the ‘tripartite synapse’ (Halassa et al., 2007a; Perea et al., 2009). During development astrocytes contribute to the formation and pruning of synapses by releasing soluble factors like thrombospondin and C1q (Christopherson et al., 2005; Stevens et al., 2007).

The roles of astrocytes in the diseased or injured CNS have been debated. Following CNS trauma, infection or inflammation astrocytes are activated, they secrete neurotoxic molecules and a process called reactive gliosis occurs (Marchetti and Abbracchio, 2005). This is followed by the formation of a glial scar. The glial scar can be detrimental for the regenerative processes taking place after injury. However, during the acute phase it is likely to serve a positive purpose by limiting the extent of injury and inflammation (Sofroniew and Vinters, 2010).

In the context of synaptic stripping the role of astrocytes is also controversial. After axotomy there is a rapid up-regulation (within days) of the intermediate filament GFAP, a classical marker for astrocytic reactivity. Astrocytic processes are then interposed between synaptic terminals and the postsynaptic membrane and according to many studies also phagocytize degenerating nerve terminals (Aldskogius et al., 1999; Bechmann and Nitsch, 1997; Wells and Tripp, 1987). The up-regulation of GFAP and VIM are considered hallmarks in astrocytic activation (Pekny et al., 1999). In an entorhinal cortex lesion model mice deficient in GFAP and VIM initially displayed fewer synaptic complexes after lesion, but 14 days post lesion the GFAP\(^{-}\)VIM\(^{-}\) mice had recovered and developed more synaptic complexes than WT mice (Wilhelmsson et al., 2004). Hence, we wanted to investigate the effect on synaptic stripping in these mice in our peripheral nerve lesion model.

### 2.4.2 Microglia

Microglia are considered to be the resident macrophages of the CNS. During the last decade numerous additional functions have attributed to this cell group. In the uninjured CNS, microglia are highly branched and their branches are highly motile displaying continuous extension and retraction (Davalos et al., 2005; Stence et al., 2001). Microglia are activated following small changes in their microenvironment (Streit et al., 1999) and the neurons themselves can instruct the microglia phenotype by secreting different factors such as CX3CL1 and CD200 and many others (Barclay et al., 2002; Ransohoff and Perry, 2009). Following activation microglia produce free oxygen radicals, nitric oxide, proteases and cytokines all of which can exert neurotoxic effects (Chao et al., 1992; Moore and Thanos, 1996; Thery et al., 1991). However, it can also exert neuroprotective effects and produce the trophic molecule TGF-\(\beta\)1 and enhance nerve repair (Streit, 2005; Streit et al., 1998).

One of many events leading to the activation and proliferation of microglia is peripheral axotomy. Within a few days of axotomy the number of microglial cells increases dramatically and glial processes
act in close proximity to the lesioned motoneurons. Ultra structural studies have demonstrated that glial processes are inserted between synaptic boutons and the motoneuron surface in a manner suggesting active synaptic removal by glial cells and presumably microglia (Aldskogius et al., 1999; Blinzinger and Kreutzberg, 1968; Chen, 1978; Sumner, 1975; Zelano et al., 2009). However, in experiments using pharmacological or genetic ablation of microglial cells no effect on the synaptic stripping event has been seen, thus indicating that other process or cell populations are crucial for the stripping of synapses (Aldskogius et al., 1999; Heppner et al., 2005; Kalla et al., 2001; Svensson and Aldskogius, 1993). Conversely, other studies demonstrate that activated microglia cells strip synapses in the cerebral cortex (Trapp et al., 2007). The vast majority of evidence however, supports the contention that the activation of microglial cells is not a prerequisite for synaptic stripping to take place.

Microglial activation and proliferation has been implicated in several neurological diseases such as Alzheimer’s disease, prion diseases, trauma and to a lesser extent Parkinson’s and Huntington’s disease (Perry et al., 2010; Streit, 2004; v Itzen et al., 1998). As a consequence, it is of great interest and importance to clarify its role in the events leading to stripping of synapses and finally neuronal death.

### 2.4.3 Schwann cells

Schwann cells are found in the PNS. There are fundamentally two types of Schwann cells, myelinating and non-myelinating Schwann cells. They have the remarkable capacity of dedifferentiating when losing contact with the axons and the molecular markers characteristic for myelinating and non-myelinating Schwann cells are down-regulated (Chiu et al., 1994). GFAP is a marker that is predominantly expressed by non-myelinating Schwann cells whereas myelin forming Schwann cells also express VIM (Jessen and Mirsky, 2005; Jessen et al., 1990). Schwann cells carry out the important job of insulating myelinated axons by forming myelin sheaths around them. Each Schwann cell forms a segment of myelin sheath of about 1mm long between the nodes of Ranvier. However, Schwann cells carry out many other crucial functions as well, such as clearance of myelin debris, providing trophic support and stabilizing the neuromuscular junctions.

After nerve injury macrophages trigger the clearance of myelin debris. Next Schwann cells ingest myelin debris (Allt, 1976) and phagocytoses myelin though to a lesser extent than macrophages (Crang and Blakemore, 1987). Schwann cells start proliferating in response to axonal and myelin debris and line up within Bands of Bunger along the basement membrane sheaths of the degenerated distal nerve stumps, which is essential for axon extension (Chen et al., 2005; Salzer and Bunge, 1980). Inhibition of Schwann cell proliferation results in severe regenerative failure (Hall, 1986). Mice lacking the two intermediate filaments GFAP and VIM have previously been shown to exhibit impaired Schwann cell proliferation and delayed nerve regeneration following nerve injury (Triolo et al., 2006).

Next, there is an intense bidirectional signaling between the Schwann cells and the lesioned axons. Schwann cells increase their expression of many neurotrophic factors such as BDNF, NGF, NT-4, GDNF and IGF-1 (Allodi et al., 2012; Heumann et al., 1987). These different neurotrophic factors exert different effects on sensory and motor axons. Other crucial molecules connected to Schwann cells and important for axon outgrowth and providing the contact between ECM and the outgrowing axons are adhesion molecules like L1/Ng-CAM, N-cadherin and integrins (Allodi et al., 2012). Axon outgrowth is however not solely dependent on any single one of these. All these events are critical for guiding the outgrowing axons to the target of innervation.

Moreover, Schwann cells are a crucial part of the NMJ. The NMJ consists of the presynaptic nerve terminal, the postsynaptic muscle fiber and the perisynaptic Schwann cell, also called terminal Schwann cells (Sanes and Lichtman, 1999). The PSCs play a crucial role in synaptic transmission as they contain many different ion channels and can both modulate synaptic transmission negatively and
positively. Moreover, PSCs are critical for long-term maintenance of the NMJ, but not for the short-term stability (5 hours) (Feng et al., 2005) and mice lacking Schwann cells are still able to form NMJs but they are not maintained over time (Morris et al., 1999). After denervation PSCs extend sprouts well ahead of the nerve terminals, and thereby guide the outgrowing axons. Also, the pattern of innervation resembles that of the earlier pattern of PSCs sprouts (Koirala et al., 2000). However, Schwann cells are not vital for the initial synapse formation, but more likely for the growth, maturation and maintenance (Woldeyesus et al., 1999).
3 AIMS

The general aim of this PhD thesis was to investigate mediators in the response within the spinal cord to peripheral axotomy, particular emphasis was placed upon molecules and cell populations that could have an influence on the process of synaptic stripping of lesioned motoneurons.

The specific aims for this thesis were:

- To study the expression of synaptic cell adhesion molecules in relation to synaptic stripping after different kinds of axonal injury and also relate the expression of these molecules to synapse restoration after regeneration.

- To identify which of the synaptic adhesion molecules SynCAM1 and members of the neuroligin family that are of relevance for the loss of inhibitory and excitatory synapses.

- To investigate the effects the absence of complement proteins C3 and C1q have upon synaptic elimination following axotomy of spinal motoneurons.

- To reveal the roles of astrocytes in the synapse elimination process following axon lesion of motoneurons by use of animals having gene deletions for vimentin (VIM) and glial fibrillary acidic protein (GFAP), which are thought to be necessary components for activation of astrocytes.

- To investigate the importance of the glial response for synaptic stripping by use of two different mouse strains (Kb^-Db^- and C3^- mice), which exhibit increased and decreased synaptic stripping respectively.

- To investigate the importance of C3 and MHC class Ia molecules for up-regulation of glial proteins and for the expression of SynCAM1, NLG-2 and -3 and NGL-2 after axotomy.
4 MATERIALS AND METHODS

4.1 EXPERIMENTAL TECHNIQUES

4.1.1 Animals used in this thesis

In papers I-II, young female Sprague- Dawley rats (B&K Universal, Stockholm, Sweden) with a body weight of approximately 200g were used. They were anesthetized with a 2:1:1 mixture of water, Hypnorm and Midazolam (2.7 ml/kg) administered intraperitoneally (i.p). A subcutaneous injection of saline (2 ml; 0.9 mg/ml NaCl) containing buprenorphine (0.05 mg/kg) provided fluid and analgesia and during surgery the rats were kept on a heating pad. The animals were allowed to survive between 3-70 days post operatively depending on the experimental requirements. When sacrificed, the animals were deeply anesthetized with i.p. pentobarbital (60mg/kg) and either decapitated or bled.

In paper III, young adult female C1q or C3 deficient mice and age matched WT mice from the same litters on a C57/B6 background were used as control. The animals were allowed to survive for a period ranging from 7-48 days.

In paper IV, adult female GFAP/VIM deficient mice and age matched WT mice on a hybrid 129/C57B6 genetic background were used. The animals were allowed to survive for a period ranging from 7-35 days.

In paper V, young adult female C3 deficient or KbDb deficient mice and age matched females on a C57/B6 background were used and allowed to survive for seven days.

In paper III all mice were anesthetized before surgery with a mixture of midazolam (Dormicum, Roche Diagnostics; 1.25 mg/ml) and Hypnorm (Janssen). The mixture was given i.p. at 0.2 ml per 25 g of body weight. In paper IV and V they were anesthetized by isoflurane inhalation. Post-operatively all animals were given a s.c. injection of saline (2 ml; 0.9 mg/ml NaCl) containing buprenorphine (0.05 mg/kg) provided fluid and analgesia and during surgery. All animals were sacrificed by lethal inhalation of CO2 in papers III-V.

4.1.2 Surgical techniques

In this thesis two variants of sciatic nerve injury were used- sciatic nerve transection (SNT) and sciatic nerve crush (SNC). In papers I-IV both methods were used and in paper V SNT was the only surgical technique used. SNC is an axotomy model where the supporting tissue- the endoneurial tubes and Schwann cell basal lamina are left intact (Haftek and Thomas, 1968)- in the nerve is preserved but the axons are severed, thereby allowing better environment for of axonal regeneration than SNT and thus giving us the possibility to study the functional recovery after axotomy. On the other hand, SNT has been studied more extensively in terms of the acute stripping of synapses after axotomy.

Generally, the sciatic nerve was dissected and visualized at mid-thigh level just below the obturator tendon. For SNT, the sciatic nerve was cut just below the obturator tendon and then otherwise left intact. In the SNC model, the nerve was crushed in the described position with a pair of forceps for 10 seconds, which has been demonstrated to result in almost complete axotomy (Lago and Navarro, 2006). After surgery all animals were clinically controlled and observed to make sure that transection had occurred.
4.1.3 Behavioral analysis

In papers I, III and IV we used a behavioral assay consisting of several different experiments measuring different abilities of the lesioned paw linked to the regeneration of the sciatic nerve, all aimed at determining the extent of functional improvement that had occurred.

First, the lesioned paw was painted with water-based paint and the mice were then placed in a tunnel on top of a sheet of paper. When the mouse had traversed the tunnel, the paper was removed and 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 representative footprints and averaged.

Secondly, for foot fault measurements, the mice were placed on a cage lid for one minute and the ratio of erroneous to correct steps was recorded.

Third, for grip strength measurements, a grip reflex was elicited by placing a metal rod on the sole of both feet simultaneously, which was then pulled away until the grip was lost. Grip ability was expressed as “no grip”, “incomplete grip” or “normal grip” compared to un-lesioned paw. Three representative foot prints or strength assessments in each animal (n=5 per group) were analysed and averaged, the average value for each animal was used for statistical analysis. All above experiments were performed blind for the respective genotypes.

4.1.4 Tissue preparation

For ISH the animal was first bled and the lumbar section of the spinal cord and/or the sciatic nerve was visualized and then rapidly dissected and trimmed. The tissue was promptly put on a small block of frozen TissueTec and frozen. Then the tissues were sectioned in a cryostat in 12 µm transverse sections at -18°C.

For IHC and EM, the animals were first transcardially perfused. For IHC the tissue was first perfused with Tyrode’s solution (20°C) followed by perfusion with fixative (20°C) containing 4% formaldehyde and 0.4% picric acid in 0.16 M phosphate buffer (pH 7.2) for 5-6 minutes. The lumbar section of the spinal cord and/or sciatic nerve was rapidly dissected and kept in the same fixative for 10 hours at 4°C. The tissues were then transferred into 0.01 M phosphate-buffered saline (PBS; pH 7.2) containing 0.1% sodium azide and stored at 4°C. One day prior to sectioning the tissue was transferred into a 10% sucrose solution (0.01 M PBS, pH 7.2, with 0.1% sodium azide) and stored overnight at 4°C. The tissue was then cut in a cryostat in 14 µm transverse sections at -18°C.

For EM, the tissue was first perfused with Tyrode’s solution at room temperature, followed by perfusion with ice-cold Tyrode’s solution. Then the tissue was perfused with fixative containing 2% glutaraldehyde in Millonig’s buffer, pH 7.4. For EM, the spinal cords were immersion fixed at 4°C and then trimmed, osmicated, dehydrated, and embedded.

In paper I, some experiments were performed on tissue also being used for ISH. The tissue was initially treated according to the procedure for ISH (see first paragraph). Before IHC, the sections were dipped in immersion fixative 3x10min at room temperature and then IHC was performed according to standard protocol.

4.1.5 In situ hybridization

Oligonucleotides were designed in Oligo 6.0. The sequences of the probes were checked in a GeneBank database search to exclude significant homology with other genes. ISH was then performed according to previously described protocol (Dagerlind et al., 1992). Briefly, the P33 labelled probes were labelled at the 3'-end with deoxyadenosine-alpha-triphosphate 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, Rochester, NY). After 3 weeks, the slides were developed in D-19 developer (Kodak), counterstained (Xylen) and coverslipped. The sections were examined in a Leica DM RBE microscope (Leica, Wetzlar, Germany), equipped with a dark field condenser and appropriate filter to examine ultraviolet fluorescence.

4.1.6 Immunohistochemistry

Sections were incubated with primary antisera, 5% donkey serum and 0.01 % PBS overnight at 4°C. Then rinsed in PBS and incubated with Cy-2, -3 or -5 conjugated secondary antibodies diluted in PBS and 0.3% Triton X-100 for 60 min at room temperature. Then rinsed 3x5 min in PBS and mounted in PBS-glycerol (1:3).

Specificity of primary antibodies was controlled with knock out tissue when deemed possible (C1q, C3, MHC, GFAP and VIM). To evaluate degree of background staining the primary antibody was omitted in the staining of control sections. For all double and triple labelling experiments sections were processed in the same fashion with additional primary and secondary antibodies.

4.1.7 Semi-quantitative measurements of ISH signal

Semi-quantitative measurements of the ISH signal were carried out as previously described (Piehl et al., 1995a). Briefly, the mRNA hybridization signal overlaying motoneuron cell bodies, identified by their size and location in the sciatic motor column, or the signal over the entire motoneuron pool was recorded. 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; Behesda, MD, USA). The grain density over the ipsilateral (IL) motoneuron pool was compared to the corresponding contralateral (CL) area in the same spinal cord section. For all experiments at least four spinal cord sections from each animal were measured and the mean IL/CL ratio for each animal was used for statistical analysis.

4.1.8 Semi-quantitative measurements of immunoreactivity

Sections were examined in a Zeiss LSM 5 Pascal confocal laser scanning microscope (Carl Zeiss GmbH, Göttingen, Germany), equipped with argon/HeNe lasers. Cy-2 and alexa-488 was visualized with 488 nm and Cy-3 with 543 nm excitation, using a 505-530 nm band pass filter (green), and a 560 nm long pass filter (red). Semi-quantitative measurements of immunoreactivity were carried out in ImageJ 1.45S (NIH, Bethesda) on confocal images at a magnification of x20, of the sciatic motoneuron pool or nerve. The immunoreactivity, i.e. number of positive pixels (area fraction) in an area containing the injured sciatic motoneuron pool in the dorsolateral area of the motor nucleus, was compared to an area of the same size containing the contralateral uninjured sciatic motoneurons in the same spinal cord section. The images were taken in the optical plane with the maximal intensity. No difference between the two methods was observed. All settings for compared images were identical.

4.1.9 Electron microscopy

Neurons with large cell bodies (>100 µm in circumference), found in the sciatic motoneuron pool and cut in the nuclear plane, were identified as motoneurons by the presence of C-type (see below) nerve
terminals. Neurons were identified as axotomized based on the occurrence of chromatolytic changes in the cell bodies, i.e disintegration of Nissl bodies with an eccentrically located nucleus.

In paper III, the synaptic terminals were classified into type F (with flattened synaptic vesicles), type S (with spherical synaptic vesicles) and type C (with a sub-synaptic cistern) according to nomenclature of Conradi (Conradi, 1969). In paper I and IV, only the number of synaptic terminals apposing the lesioned motoneuron was investigated. Two motoneurons in each animal were studied and the average number of boutons per 100 µm cell membrane was used for statistical analysis.

4.2 METHODOLOGICAL CONSIDERATIONS

4.2.1 Species differences

In this thesis, we have used both rats and mice, and also several different kinds of knock-out mice. This complicates the whole picture and makes comparisons between papers difficult, as there are some major differences between the species and potential differences between mouse strains. In papers I and II we used rats as our experimental model, and in papers III-V we used mice to enable us to study the influence of different protein deficiencies.

First of all, there are obvious anatomical differences between rats and mice. The sciatic motoneuron pool forms an elongated nucleus that extends from L3-L6 in rat (Swett et al., 1986) and L3-S1 in mice. (Rigaud et al., 2008). About 2000 sciatic motoneurons make up the rat sciatic motor column and about half as many in the mouse (Rigaud et al., 2008; Swett et al., 1986) and these innervate the hind limb muscles. However, in that respect anatomical differences should not affect our results, since up-regulation of GAP-43 for ISH experiments and the up-regulation of glial markers for IHC were used as controls throughout all experiments.

After sciatic nerve lesion approximately 30% of the lesioned motoneurons undergo apoptosis in mice, in rats a less severe reaction occurs. This number increases the closer to the soma the axons are lesioned (Lowrie and Vrbova, 1992; Moran and Graeber, 2004). However, these numbers vary greatly between different species, age groups and different studies. In rats, survival rates between 70-100% have been described following peripheral axotomy (Yamada et al., 2011; Yu, 1988) and in mice these numbers varies between 25-90% (Oh et al., 1994; Yamada et al., 2008). Obviously, the distance from lesion site to target innervation as well as from lesion site to motoneuron soma will be less in mice compared rats due to the difference in size. This will lead a greater inflammatory response in the spinal cord in mice compared to rats. Secondly, the lesioned axon will need to grow a smaller distance in order to re-innervate target tissue in mice leading to faster functional recovery. In our studies we have not detected any obvious difference in terms of the degree of synaptic stripping taking place.

4.2.2 In situ hybridization

In situ hybridization is an excellent method for visualizing relatively small amounts of mRNA. However, it offers some limitations. It cannot compete with methods like PCR for detecting extremely small amounts of mRNA since in situ hybridization always results in some background staining. Compared to PCR, in situ hybridization only offers the possibility for semi-quantification and not absolute numbers. When comparing IL to CL side, it is therefore extremely important to ensure even staining over tissue. The staining depends heavily on tissue storage time, temperature during incubation and emulsion thickness. To ensure that these variables were constant tissues were compared with other sections from the same experiment. Furthermore, comparison of ISH signal over one side to signal over the adjacent side was always done within the same tissue sections. Therefore, studied areas should have been exposed to identical conditions.
Another important issue is the specificity of the probes. These were designed and then controlled in a gene bank by a database search to exclude significant homology with any other mRNAs. Moreover, for every studied molecule two different probes were designed with different sequences. The probes were compared and it was ensured that they rendered similar staining patterns. The probes were also controlled in tissues with known expression of the target mRNA.

### 4.2.3 Immunohistochemistry

For IHC there are many obstacles regarding specificity of antibodies and many possible control experiments to perform. First of all we have tried to use well-characterized antibodies and for some antibodies we have performed verifying western blots. For GFAP, VIM, C3, C1q antibodies were tested on null mutants in order to exclude cross-reactivity. Only antibodies rendering completely negative results compared to WT were used. For antibodies where no null mutant mice were available we tested antibodies on different tissues with known expression of respective protein and performed secondary incubation in the presence and absence of primary antibody. Secondary antibodies were also incubated in the presence and absence of primary antibodies in order to exclude any unspecific staining.

### 4.2.4 Electron microscopy

Electron microscopy is an excellent method to study synaptic stripping in detail with non-semiquantative measurements. However, the method offers some limitations as well. Compared to IHC where we studied synapse elimination in the whole sciatic motoneuron pool including the dendritic tree, EM only gives the possibility to study synaptic stripping on individual motoneurons, resulting in a good picture of what is happening on the surface of the individual motoneurons, but not in the surrounding milieu. Note, that generally in our studies the degree of stripping was much more severe when studied by EM than IHC. This is in line with what we expected, since previous studies have demonstrated that elimination of synapses is generally much more severe from the motoneuron soma compared to that from the dendritic tree (Brannstrom and Kellerth, 1998).

Furthermore, as a prerequisite for synapses to be included in our EM quantification analysis they need to be directly apposing the motoneuron soma. Considering the extremely thin slices used in the EM analysis some of the synapses will not be cut exactly in the plane where they are apposing the soma. Next, EM quantification of synapses is based on certain morphological appearances i.e. postsynaptic density, c-boutons, synaptic vesicles and directly apposing membranes. After axotomy the motoneurons and synapses undergo morphological changes (Blinzinger and Kreutzberg, 1968; Chen, 1978), which might lead to an underestimation of synapse numbers. Collectively this might lead to an underestimation of synapse numbers.

Classifying synapses into type F, type S and type C boutons offers great possibilities, but also some difficulties. First, the tissue handling is of great importance- from perfusion to sectioning. If not optimized, vesicles usually appearing flattened after perfusion might turn up as elliptical shaped making them impossible to classify according to this paradigm. There are also other bouton types on the dendritic tree- the M-boutons- that will not be included in this classification. Thus, this experimental setup will only render a limited picture of what is going on.

### 4.2.5 Confocal microscopy

Confocal microscopy was first developed in 1951 by Naora (Naora, 1951, 1955). However, it was not until 1985 that a confocal microscope somewhat similar to the ones existing today was developed. The
Key features of the confocal microscope are the light source (lasers), focusing on a limited spot by the objective lens, emitted light from the excited fluorophores is focused to a detector via a pinhole excluding light out of focus and thus creating a picture with great resolution. By scanning several focus planes of a slice three-dimensional images can be created and analysed. When utilizing a complex technique with many adjustable variables such as this, one can run into different difficulties.

The first problem is the risk of a false positive signal. Since the detectors are so sensitive, and their settings are subject to personal bias, it is almost always possible to acquire some signal. To prevent false positive signal, the background level was set by using null mutant mice when deemed possible and when not possible the primary antibody was omitted and only the secondary antibody applied. These control samples were analysed with same settings as the experimental samples and adjusted so that no background signal was seen.

Second, in our experiments one should remember that we have only performed semiquantitative analysis and not quantified absolute protein amounts. For absolute quantifications western blot or PCR should be used. There can be great variations in signal intensity over the same slide resulting in false positive differences. Therefore, measurements were only performed on one part of the spinal cord with the immunoreactivity detected with the exact same microscope settings in a different part of the same spinal cord section. Signal intensity can also distort the results; saturated signal or extreme weak signal will lead to altered results. To prevent this microscope settings were adjusted to give signal intensity in a predefined range.

Third, in terms of co-localization analysis there are several difficulties. In many cases the proteins being studied are small and closely related. To eliminate these risks, we performed Z-stacks, used small pinholes, high-resolution images and optimized microscope settings. Still, especially regarding in depth analyses there are limitations in the resolution that can be reached and these should be taken into account when analysing small molecules.
5 RESULTS AND DISCUSSION

5.1 ADHESION MOLECULES INVESTIGATED IN THIS THESIS

5.1.1 SynCAM1 and 2

In paper I we examined mRNA levels of SynCAM1 and 2 in spinal motoneurons after two different types of nerve lesion. SynCAM1 is an adhesion molecule with synapse inducing properties (Biederer et al., 2002) and SynCAM2 also has the capability to induce synapses and mediates synaptic adhesion (Fogel et al., 2007). Initially, we confirmed previous findings that SynCAM1 mRNA levels are down-regulated already three days after axotomy to approximately 30% of what was seen on the contralateral side at a time when no loss of synaptophysin is seen (Zelano et al., 2007a). This was followed by a down-regulation of SynCAM IR on the surface of the lesioned motoneurons. However, we could not detect any difference in SynCAM2 mRNA expression levels.

Next, we wanted to study SynCAM1 during a phase of regeneration where synapses return to the lesioned motoneurons. SNT undoubtedly results in axotomy, however the degree of regeneration occurring after SNT is highly variable, so instead of SNT we performed SNC. SNC is a method where the surrounding perineurium is left intact and the lesioned axons are therefore given a chance of regenerating down the same path as they inhabited before lesion (Lago and Navarro, 2006). In the acute phase both lesion models turned out similar in terms of down-regulation of synaptophysin IR and SynCAM1 mRNA levels. In the SNT model we detected a significant correlation between synaptophysin IR and SynCAM1 mRNA levels. After SNC the synaptophysin IR levels returned to starting values, as did SynCAM1 mRNA expression levels.

To summarize, these data indicate that SynCAM1 mRNA expression reflects synapse number on spinal motoneurons and that SynCAM1 expression is not influenced by the quality of the contact with the distal stump, since no difference was seen between the SNT and SNC experiment. This fits well with a recent report that levels of SynCAMs are dynamic in the development of the spinal cord, suggesting a role for SynCAMs in establishing synaptic connections (Thomas et al., 2008).

5.1.2 Neuroligins -2 and -3

The neuroligins are a family of postsynaptic adhesion molecules that exhibit synapse-inducing properties, but lately it has been demonstrated that they are more involved in synapse function and maturation than just mere synapse number. NLG-2 is located exclusively to inhibitory synapses while NGL-3 locates to both excitatory and inhibitory ones. They are both down-regulated after sciatic nerve lesion (Zelano et al., 2007b).

In paper I, we studied the long-term regulation of NLG-2 and -3 during the acute and regenerative phase after sciatic nerve transection and crush. We first demonstrated the presence of NLG protein in the spinal cord. Next we studied the mRNA expression levels following SNT and SNC. Both NLG2 and 3 decreased acutely following nerve lesion, however less dramatically in the SNC group compared to the SNT group. In the long run, the ISH signal levels were restored for both groups, but a slight overshoot was seen for NLG3 (125% of control values). The ISH signal levels did not seem to correlate to synapse number. The loss of synapses were the same in the acute phase for the SNC and SNT groups, but highly different in the long term perspective, where we observed a complete recovery in synapse number in the SNC group, but a highly variable one in the SNT group. We performed a correlation analysis of these adhesion molecules, which showed that NLG2 mRNA levels were correlated
to synaptophysin IR in the SNC experiment and a negative correlation was seen between NLG3 and the excitatory synapse marker VGLUT2.

These data are not as easily interpreted as the data from the SynCAM1 experiment. Clearly these molecules do not seem to be mere regulators of synapse numbers, but play other intricate roles. Several studies following the first ones, where it was proposed that NLGs act as a synapse inducing molecule, have argued that NLGs are more involved in synapse function than mere synapse numbers.

5.1.3 Netrin G-2 ligand

NGL-2 is a postsynaptic adhesion molecule binding to its presynaptic binding partner β-neurexin and in vitro has been shown to exhibit synapse inducing properties with a bias for excitatory synapses. (Kim et al., 2006). As synaptic detachment from axotomized motoneurons preferentially involves excitatory inputs we found it of interest to study NGL-2 in our lesion model (Lindå et al., 2000). In paper II, we performed SNT or SNC and studied the mRNA expression levels of NGL-2 in lesioned motoneurons.

We observed a clear correlation between loss of synaptophysin IR and down-regulation of NGL-2 mRNA levels. Following regeneration in the SNT model, NGL-2 levels increased but were not completely restored. Contrastingly, in the SNC model where synapse restoration is complete 10 weeks post lesion NGL-2 expression levels were also restored. Hence, we conclude that NGL-2 expression levels correlate to synapse covering, but the existence of NLG-2 protein at synapses still needs to be proven.

5.2 COMPLEMENT C1Q AND C3

Complement C1q is the initiating protein of the classical complement cascade and has recently been shown to play a role in synaptic pruning during development via ‘tagging’ of synapses (Stevens et al., 2007) and is perceived as particularly important for the elimination of excitatory synapses (Chu et al., 2010).

In paper III, we observed a vast up-regulation of C1q mRNA and protein around the lesioned motoneurons one week after axotomy. C1q mRNA expression displayed a pattern corresponding to that of activated microglia. C1q protein was more difficult to localize to any specific cell or cellular compartment, but some IR was seen in close interaction with the motoneuron soma, but did not clearly co-localize with synaptophysin positive punctae. C3 mRNA was difficult to localize after axotomy in the spinal cord, but has previously been shown to be up-regulated after peripheral nerve lesion. C3 IR was however distinctly up-regulated one week after nerve lesion and co-localized preferably to astrocytes and to a lesser extent to microglia. In vivo both microglia and astrocytes are able to express C1q and C3 fig.2 (unpublished observations, Berg and Plantman).

Next we wanted to test the hypothesis whether C1q and C3 are important for synaptic stripping or not. The absence of C1q protein did not have any effect on the synaptic stripping after axotomy measured by IHC for synaptophysin, VGLUT-2 and VIAAT. Thus, we did not proceed with investigating the stripping response by electron microscopy. However, for C3−/− mice we observed a hampered stripping response following axotomy. Synaptophysin IR was decreased to a greater extent over the sciatic motoneuron pool in WT mice compared to C3−/− mice. The immunoreactivity for the excitatory synaptic marker VGLUT-2, displayed a similar down-regulation in both WT and C3−/− mice. VIAAT, the marker used for inhibitory synapses, displayed a profound down-regulation in WT, but not in C3−/− mice. In order to test these results further we performed electron microscopy on lesioned motoneurons and analysed the types of boutons that were removed. The S-type boutons—i.e. excitatory synapses—were eliminated to a similar extent in both WT and C3−/− mice. The F-type boutons—i.e. inhibitory synapses—were however,
eliminated to a much smaller degree in the C3⁻/⁻ mice compared to WT mice. Thus, this experiment confirms previous data from the IR quantification experiment showing that the absence of complement C3 results in hampered synapse elimination following peripheral axotomy. As previously described, the stripping response might be a way for the lesioned motoneurons to redirect its resources from a transmitting neuron to a regenerating one.

**Figure 2:** *In vitro* expression of complement C1q and C3. In culture astrocytes express high levels of C1q and slightly lower levels of C3. Microglia express both C1q and C3 to a fairly similar extent.

Next we asked how this impeded stripping of synapses would affect regeneration. First, we examined the up-regulation of the regenerative associated gene GAP-43 by ISH. Following axotomy we observed a massive up-regulation of GAP-43 in both WT and C3⁻/⁻ mice, however this increase was about twofold in the C3⁻/⁻ mice compared to WT mice. To further assess the functional significance of this difference we performed a set of behavioural studies on these mice. Instead of SNT we performed SNC, which results in almost complete regeneration and reinnervation compared to SNT. We tested grip strength, toe spread and grid walk. For toe spread and grid walk we could not detect any differences between the strains. However, for grip strength we were surprised to see that C3⁻/⁻ mice regained a complete recovery about 4 days before WT mice did. Both strains regained an insufficient grip at the same time. At this time we still observed similar levels of VGLUT2 IR in the spinal cord, but higher levels of synaptophysin and VIAAT IR.

Complement is a crucial component of the inflammatory response after nerve injury, and is highly up-regulated after axotomy at the lesion site (Dailey et al., 1998; Ohlsson et al., 2003; Ramaglia et al., 2008). We assessed the degree of inflammation over the lesion site by CD68 and Iba1 IR and could not detect any difference. Conversely, many other studies have shown that inflammatory process around the lesioned nerve is affected in C3⁻/⁻ mice and that complement C3 is crucial for the recruitment of macrophages (Dailey et al., 1998; Ramaglia et al., 2008). It should be noted that in those studies complement has primarily been described as a crucial factor for the initial recruitment of macrophages.
C6\textsuperscript{\textminus} rats displayed an initially delayed Wallerian degeneration but the axonal and myelin degradation was normal one week after lesion and that is the time-point that we studied.

Furthermore, we wanted to see whether there was any delay in the re-innervation of the neuromuscular endplates. This was performed by counting the number of synaptophysin-positive motor endplates. The results indicate that the behavioural difference we observed was not due to peripheral effects i.e. altered axonal regeneration, but presumably due to the preservation of central synapses. To some extent these data contradicts previous results and theories from Lindå et al. where it was argued that the synaptic stripping response protects motoneurons from excitotoxicity (Lindå et al., 2000). However, in our experiments, the excitatory synapses are still eliminated to the same extent in WT and C3\textsuperscript{\textminus} mice and it is only the inhibitory synapses that are spared. Thus, it can be assumed that the motoneurons in C3\textsuperscript{\textminus} mice are also kept in an inhibitory state that is beneficial for regeneration.

Moreover, previous results from Dailey et al. (Dailey et al., 1998) showed that rats that were depleted of serum C3 exhibit a delayed onset of regeneration. One should however note that it in our model complement is absent both at the lesion site and in the spinal cord. There are several other issues one has to take into account for when comparing these studies i.e. different species and temporal resolution.

To summarize our findings, we observed a quicker functional recovery for C3\textsuperscript{\textminus} mice and this was accompanied by hampered synaptic elimination of preferentially inhibitory synapses. No other difference was seen. The most intriguing question is still how the stripping of synapses occurs. Is it because of a ‘tagging’ process as proposed by the Stevens et al. (Stephan et al., 2012; Stevens et al., 2007) from studies in the visual system? Or is it due to other reasons like disturbed chemo attraction of inflammatory and glial cells, or changed gene expression of these cells? In our studies we observed a halo of C1q-IR, which could not be linked with any cellular structure but rather appeared to have an extracellular position. Its role is unclear but we found no evidence that it played any role in mediating synapse stripping in our model.

5.3 GLIAL ROLE IN SYNAPTIC STRIPPING AND NERVE REGENERATION

5.3.1 Astrocytes

Astrocytes are the most abundant cell type of the mammalian CNS. However, their function is just in the beginning of being unraveled. Historically, they were seen as a ‘nerve glue’ with primarily a passive role at the synapse (Somjen, 1988), however, later on other functions have been attributed to them, like regulating ion concentrations, taking up neurotransmitters and providing substrates for energy metabolism (Sofroniew and Vinters, 2010). Recent advances in glial research have demonstrated many more sophisticated roles for astrocytes and a great importance for these cells in the process of synaptogenesis (Pfrieger and Barres, 1997; Wilhelmsson et al., 2004).

In paper IV we examined mice deficient in GFAP and VIM; two intermediate filaments necessary for astrocyte function. One week after nerve lesion we observed a vast up-regulation of GFAP and VIM IR in the spinal cord. In terms of synapse loss we could not detect any difference between WT and GFAP\textsuperscript{\textminus} VIM\textsuperscript{\textminus} assessed by IHC. However, when we only studied the motoneuron soma by electron microscopy, where the degree of stripping is the most severe, we observed a hampered loss of synapses after axotomy. Furthermore, these mice exhibited a slower functional recovery after axotomy assessed in several different behavioral assays and also slower regeneration of lesioned axons. These results should however be interpreted carefully since GFAP and VIM are both present in Schwann cells as well, which are crucial for axon regeneration.
In the next study (V), we studied the GFAP IR up-regulation after sciatic nerve lesion in two different mouse lines C3 or MHC class Ia molecule deficient mice. These two strains exhibiting contrasting responses to axotomy in terms of synaptic stripping. However, the degree of GFAP up-regulation was similar in both strains. Thus, we conclude that none of these immune molecules are crucial for the up-regulation of astrocytes after axotomy and that other signaling pathways are involved.

5.3.2 Microglia

Microglia are clearly up-regulated shortly after peripheral nerve lesion and interact closely with the lesioned motoneurons (Blinzinger and Kreutzberg, 1968). Thus, it has been proposed that they are involved in the removal of synapses following axotomy. Other studies, however, have shown that blockade of microglial activation does not seem to effect the extent of synaptic stripping (Svensson and Aldskogius, 1993). Although their interaction with neurons after axotomy has been known for more than 40 years their exact role in the process of synaptic stripping is still not clear.

Our lesion models result in vast activation of microglial cells both in rats and mice. Since microglia have been implicated in synaptic stripping, we decided to investigate the degree of activation and up-regulation of microglia in two different mouse lines with contrasting response in terms of synaptic stripping to peripheral axotomy (paper V). Mice deficient of the MHC class Ia molecule, so-called K\textsuperscript{b/c} D\textsuperscript{b/c} mice exhibit an augmented degree of synaptic stripping after nerve lesion. In contrast, complement C3\textsuperscript{-/-} mice exhibit a reduced degree of synaptic stripping following nerve lesion. In our studies we could not detect any difference in the up-regulation of microglia by the use IHC, indicating that C3 or MHC class Ia do not affect the glial reactivity. Presumably, microglia is still involved in the removal of synapses, but there are other mechanisms instructing which synapses are destined to be removed. Mere activation of microglia does not seem to determine the extent of synaptic stripping.

5.3.3 Schwann cells

In paper IV, we briefly investigated the Schwann cell response following peripheral axotomy in WT and GFAP\textsuperscript{-/-}/VIM\textsuperscript{-/-} mice. Within a few days after axotomy we observed an up-regulation of GFAP and VIM over the lesioned nerve, which slightly started to decrease after a week. Since the behavioral recovery was delayed in the GFAP\textsuperscript{-/-}/VIM\textsuperscript{-/-} mice we hypothesized that the reaction of Schwann cells might be affected. Subsequently, we studied IR for the Schwann cell marker S100, which is expressed by both myelinating and non-myelinating Schwann cells. The IR for S100 was clearly decreased in GFAP\textsuperscript{-/-}/VIM\textsuperscript{-/-} compared to WT mice indicating a slower reestablishment of Schwann cells in the GFAP\textsuperscript{-/-}/VIM\textsuperscript{-/-} group. This could partly explain the delayed recovery. Furthermore, Schwann cells are a crucial component in the re-myelination process and we therefore studied the expression of the myelin marker, MBP in both groups. Here, we could not detect any difference between the strains, although a trend was seen towards higher expression in WT mice. This indicates that the myelination process is primarily not affected in these mice.

5.4 ADHESION MOLECULES IN MICE EXHIBITING DIFFERENT DEGREE OF SYNAPTIC STRIPPING

In paper III, we describe decreased synaptic stripping in a mouse strain lacking complement C3 (Berg et al., 2012). This is opposite to the findings in mice lacking functional MHC class I molecules, which display an increased synaptic stripping response to axotomy (Oliveira et al., 2004b). In paper V, we
asked whether the expression of the previously studied synaptic adhesion molecules in motoneurons, reflects the altered synaptic stripping that is seen in these two mouse strains.

Either the expression levels of the synaptic adhesion molecules follow the synapse number or not. The first would indicate that the expression levels of synaptic adhesion molecules are either a consequence of synapse removal or a final common pathway in the process of synapse elimination. The second alternative would demonstrate that C3 and MHC class I molecules exhibit their effects via pathways independent of these adhesion molecules.

We performed ISH for SynCAM1, NLG-2 and -3 and NGL-2 seven days after axotomy. Both K\(^{\beta}\)\(^{-}\)D\(^{\beta}\)\(^{-}\) and C3\(^{-}\) mice displayed a response, which was very similar to what was seen in WT mice i.e. a general down-regulation of the studied adhesion molecules in terms of mRNA expression levels. Thus, we conclude that it does not by itself infer a downsizing of the synaptic connectivity with lesioned neurons and that the down-regulation of these adhesion molecules is a neuronal response independent of C3 and MHC-I molecules.
5.5 CONCLUSIONS

In this thesis we have examined the microenvironment around lesioned motoneurons with regards to molecules and cells of potential importance for stripping of synapses. The thesis provides new evidence that complement C3 play a crucial role in the synaptic stripping process and this effect is not mediated via the adhesion molecules SynCAM1, NLG-2 and -3 and NGL-2. Furthermore, glial activation does not seem to be the determining factor for this phenomenon.

Specifically we have shown that:

- The adhesion molecules SynCAM1, NLG-2 and -3 and NGL-2 are all down-regulated in lesioned motoneurons before synaptic stripping occurs and the quality of the contact with the distal stump does not seem to influence the stripping process in the acute phase. SynCAM1 expression is correlated to restoration of synaptophysin IR and NGL-2 expression also shows a pattern correlating to the restoration of synaptophysin IR. The expression of mRNA encoding NLGs on the other hand seem to depend on the contact with the distal stump i.e. good contact enhances NLG expression. The NLGs seem to have a greater role in synapse function rather than actual numbers.

- Complement proteins C1q and C3 are both up-regulated following SNT and SNC. Complement C3 deficient mice exhibit a hampered stripping process. However, absence of the upstream complement protein C1q does not affect this phenomenon. Thus, we conclude that this effect is mediated via a non-classical complement pathway. C3-/ mice also show increased GAP-43 expression following axotomy and faster functional recovery, but no signs of affected inflammation at the site of lesion.

- Mice lacking the two intermediate filaments GFAP and VIM displayed a slightly smaller removal of synaptic boutons compared to WT mice. We suggest that this might indicate that reactive astrocytes up-regulating GFAP and VIM take part in the synaptic removal process. Furthermore, these mice display a delayed onset of regeneration. This is most likely due to unfavourable effects on Schwann cell proliferation rather than affected degree of synaptic stripping, since mice exhibiting augmented synaptic stripping display delayed recovery of hind limb function (Oliveira et al., 2004a) and mice displaying hampered degree of synaptic stripping regain function in the lesioned hind limb faster (Berg et al., 2012).

- There is no difference in expression levels of SynCAM1, NLG-2 and -3 and NGL-2 when comparing C3-/ mice to Kb/-Db/- mice. These are two mouse strains showing completely different response to axotomy. Synaptic stripping is enhanced in Kb/-Db/- and hampered in C3-/ mice. Thus, we conclude that the degree of stripping is dependent upon processes additional to the down-regulation of these adhesion molecules.

- Glial activation as measured by IR for Iba1- a marker for microglia- and GFAP- a marker for astrocytes- is similar in C3-/ and Kb/-Db/- mice. Therefore we conclude that the up-regulation of microglia and astrocytes is not dependent on complement C3 nor MHC class I molecules. Furthermore, we conclude that glial activation is not the only factor determining the degree of stripping, but other more precise molecular pathways are involved, presumably complement C3 and MHC class I molecules.
6 DISCUSSION AND FUTURE EXPERIMENTS

In this thesis we have investigated certain immune and adhesion molecules in relation to the synaptic stripping response occurring after peripheral nerve lesion and also the importance of glial molecules in this process. However, there are still many unanswered questions.

Regarding the adhesion molecules SynCAM1, NLG-2 and -3 and NGL-2 we have demonstrated that they are all down-regulated before synaptic stripping occurs. However, expression levels of these adhesion molecules are not clearly correlated to the degree of synaptic stripping taking place. Especially, for the NLGs there are many exciting functions that are not fully understood and most likely many other adhesion molecules remain to be discovered which could have synapse inducing properties (Brose, 2009). Neither does the expression of the adhesion molecules NLGs -2 and -3 or SynCAM1 and NGL-2 seem to be affected in the C3<sup>-/-</sup> and Kb<sup>-/-</sup>Db<sup>-/-</sup> who exhibit different degrees of stripping after axotomy. Gaining understanding of how the neuroligins affect synapse function could in the long run lead to a better understanding of many diseases in the autism spectrum field.

Next, it still remains unclear how complement C1q and C3 are involved in synapse elimination. The ‘tagging’ hypothesis is appealing, but in our experiments we could not observe any clear signs of such a process. Imunno-EM is an excellent method with which to further elucidate this matter and is one of the best available methods to see if such a tagging occurs or not in high resolution. However, it offers many limitations and many new techniques are evolving which allow study of synapse elimination in real time, e.g. by the usage of two-photon confocal microscopy. Next, it is still not clear how the inflammatory process at the lesion site is affected by complement signalling and most likely complement factors exert different actions in the acute and chronic phase. Thus, in coming experiments it will be of great interest to elucidate precise spatiotemporal functions of specific complement components.

In our studies we have used relatively crude measurements of inflammation and we have not investigated if the re-myelination process is disturbed. Conducting neurophysiology on lesioned nerve and measuring signal speed could answer this and would further indicate whether there are peripheral or central effects leading to the behavioural phenotype. The development of conditional knockout mice can make this task possible and complement C3 can be knocked-out specifically in macrophages, microglia, astrocytes and motoneurons for example. Maybe the most intriguing question is via which pathways complement C3 does exert its actions? Is it acting at one particular complement receptor? If that is the case, would it then be possible to target and block this receptor and then prevent the stripping process and in the long run modify disease progression? Would this be detrimental or beneficial for axon regeneration and neuronal survival?

Next, the activation of astrocytes and microglia does not seem be influenced by the absence of complement C3 or the MHC class I molecule. This also indicates that the mere up-regulation and activation of microglia and astrocytes is not the determining factor for the degree of synaptic stripping taking place. However, the GFAP<sup>-/-</sup>VIM<sup>-/-</sup> mice that presumably lack reactive astrocytes display a less severe stripping response following axotomy compared to WT mice. Presumably, the contribution of astrocytes to the process of synaptic stripping is so subtle that no clear difference is seen in the experiment with C3 and MHC class I molecule deficient mice.

There are many ways to manipulate the stripping response e.g. C3<sup>-/-</sup> mice exhibit less stripping of synapses (Berg et al., 2012), MHC class Ia molecule deficiency results in an augmented stripping response (Oliveira et al., 2004a), and administration of neurotrophins, which hampers the stripping response (Davis-Lopez de Carrizosa et al., 2009). However, in order to finally be able to elucidate the roles of microglia, astrocytes and adhesion molecules, conditional knockout mice are most likely...
required. In all probability though, synaptic stripping is not a result of the actions of a single cell, but supposedly a complex interaction between different cell types and molecules.

From a clinical perspective it is known that synaptic stripping is an early event in neurological diseases such as Alzheimer’s disease, multiple sclerosis and prion diseases (Marques et al., 2006; Selkoe, 2002; Zhu et al., 2003). Most likely the disease process is already started at this stage, but it has been argued that if the stripping response can be stopped or delayed, then the progress of the disease could be slowed down. In these diseases there is a general inflammatory process occurring, with increased complement expression and proliferation of glial cells. If this can be detected and treated before stripping of synapses occur, then disease progress could be arrested. Additionally, it has not been investigated how the expression levels of the synaptic adhesion molecules described in this thesis are affected in diseases such as Alzheimer’s disease. Maybe, they are also down-regulated in this case before synaptic stripping occurs. Investigation of these events could lead to the possibility of more rapid and improved disease detection. Furthermore, an enhanced understanding of the signals critical for retaining lost synapses could lead to improved strategies for slowing of disease progression.
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