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Immune Recognition Molecules in Synaptic Plasticity and Regeneration of Spinal Motoneurons

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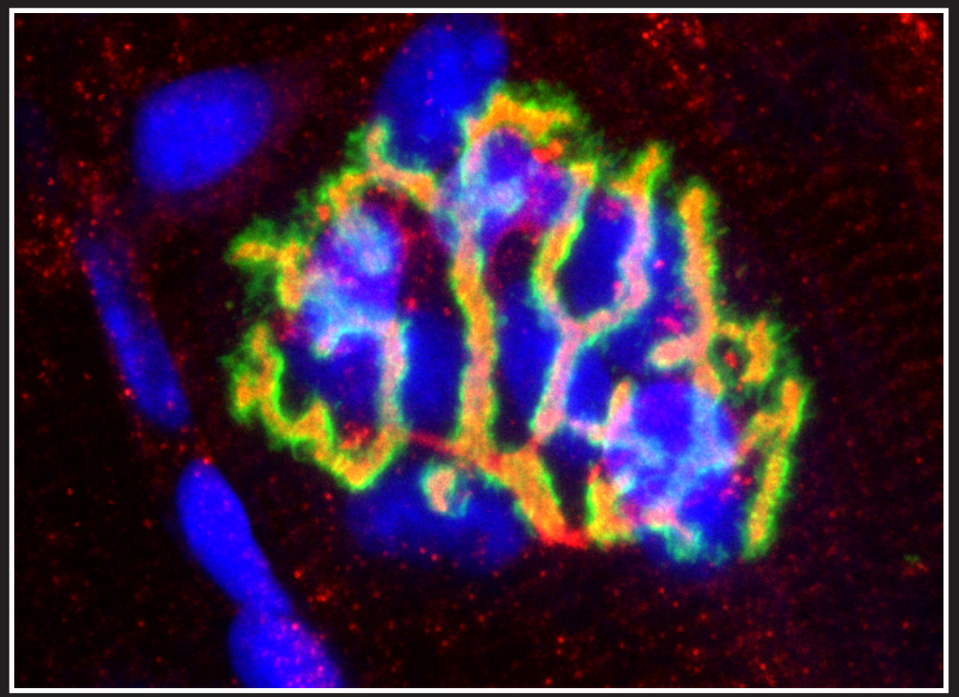

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Cover illustration: A confocal maximum projection micrograph of a mouse neuromuscular junction stained with S100 in red, α-bungarotoxin in green and TOTO-3 in blue.

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ABSTRACT

This thesis is based on the emerging concept that pattern recognition molecules, originally characterized in the immune system, may be expressed and used by neurons to mediate also non-immune functions. In line with this concept, the major histocompatibility complex (MHC) class I and certain complements proteins have been implicated in synaptic plasticity in the developing visual system.

In **Paper I**, we studied the expression of MHC class I mRNAs and proteins in normal and axotomized mouse spinal motoneurons. Two mRNAs encoding classical MHC class I molecules (H2-K^b and H2-D^b) were moderately expressed in uninjured motoneuron cell bodies. After a peripheral nerve lesion, both mRNAs were strongly up-regulated by axotomized motoneurons and surrounding glial cells. Using a MHC class I antibody with affinity for H2-D^b, we observed moderate immunoreactivity (IR) in the cell bodies of a subpopulation of uninjured spinal motoneurons. After a peripheral nerve lesion, H2-D^b IR was strongly increased in activated microglia. In contrast to the *in situ* hybridization results the H2-D^b IR remained unchanged in axotomized motoneuron cell bodies. We then further investigated the motoneuron *in vivo* expression of H2-D^b in the periphery. H2-D^b IR was detected in a subpopulation of axons in the sciatic nerve and at the presynaptic side of the neuromuscular junction (NMJ) in hind limb muscles. When studying mice deficient in classical MHC class I ($K^{b-/-}D^{b-/-}$), we observed abnormal dynamic changes in NMJ density during muscle reinnervation and delayed motor recovery after a sciatic nerve crush (SNC). During the reinnervation phase, $K^{b-/-}D^{b-/-}$ mice also displayed an attenuated proliferation of terminal Schwann cells at NMJs compared to wild-type mice (WT). Interestingly, we found expression of the paired immunoglobulin receptor B in dissociated Schwann cells and histological sections from the sciatic nerve.

In order to investigate the role of MHC class I proteins in central motoneuron plasticity, we studied elimination of synaptic contacts from axotomized motoneuron cell bodies at the ultrastructural level in **Paper II**. In contrast to a previous publication by Shatz et al. 2000, axotomized motoneurons in mice lacking functional MHC class I ($TAP1^{-/-}$ and $\beta_2m^{-/-}$) displayed an increased synaptic elimination compared to WT mice. Moreover, in $\beta_2m^{-/-}$ mice the remaining terminals were randomly dispersed along the cytoplasmic membrane in difference to WT animals where they were tightly clustered. When analyzing the types of synaptic terminals that were retracted in the $\beta_2m^{-/-}$ mice, we found a preferential loss of inhibitory terminals. In parallel, axonal regeneration appeared to be hampered in the absence of functional MHC class I molecules.

Since complement-deficient animals ($C1q^{-/-}$ and $C3^{-/-}$) are shown to display a phenotype resembling that of MHC class I-deficient mice regarding synaptic plasticity in the visual system, we investigated the role for complement proteins in adult motoneuron plasticity in **Paper III**. In accordance with a previous study by Steven et al. 2007, $C3^{-/-}$ animals displayed a diminished reduction in synapse density and covering of axotomized motoneurons. The histological expression pattern of C1q and C3 in the spinal cord was somewhat hard to interpret. We found a clear up-regulation of complement mRNA and protein in the axotomized sciatic motor pool, but we have so far failed to determine the subcellular localization with certainty. Nonetheless, we found complement IR in close association with the motoneuron surface and with presynaptic terminals on proximal dendrites and with surrounding glial cells. In addition, $C3^{-/-}$ animals recovered their motor function more rapidly after a SNC.

In conclusion, we have investigated and found evidence of new roles for classical immune molecules in motoneurons with regard to synaptic plasticity and regeneration. The subcellular expression and signalling pathways remain to be described before specific functions and sites of action for these molecules can be determined. Further studies of neuronal immune molecules will be important in order to gain insight into the mechanisms of cellular interaction between different types of neurons or glial cells.

LIST OF PUBLICATIONS

The thesis is based on the following publications, which will be referred to in the text by their roman numerals:

I. **Sebastian Thams**, Petter Brodin*, Stefan Plantman*, Robert Saxelin, Klas Kärre and Staffan Cullheim. Classical MHC Class I Molecules in Motoneurons – New Actors at the Neuromuscular Junction. Journal of Neuroscience 2009, Oct 28;29(43):13503-13515.

II. Alexandre LR Oliveira*, **Sebastian Thams***, Olle Lidman, Fredrik Piehl, Tomas Hökfelt, Klas Kärre, Hans Lindå and Staffan Cullheim. A role for MHC class I molecules in synaptic plasticity and regeneration of neurons after axotomy. Proc Natl Acad Sci (PNAS) USA. 2004 Dec 21;101(51):17843-8.

III. Johan Zelano*, Alexander Berg*, **Sebastian Thams**, Marcella Pekna, Milos Pekny och Staffan Cullheim. Reduced loss of synapses on spinal motoneurons parallels more rapid motor recovery after sciatic nerve lesion in complement C3-deficient mice. Manuscript.

*Equal contribution.

OTHER PUBLICATIONS BY THE AUTHOR NOT INCLUDED IN THE THESIS:

Wilhelm Wallquist, Manuel Patarroyo, **Sebastian Thams**, Thomas Carlstedt, Birgit Stark, Staffan Cullheim and Henrik Hammarberg. Laminin chains in rat and human peripheral nerve: distribution and regulation during development and after axonal injury. J Comp Neurol. 2002 Dec 16;454(3):284-93.

Wilhelm Wallquist, Stefan Plantman, **Sebastian Thams**, Jill Thyboll, Jarkko Kortesmaa, Jan Lännergren, Anna Domogatskaya, Sven Ove Ögren, Mårten Risling, Henrik Hammarberg, Karl Tryggvason, and Staffan Cullheim. Impeded interaction between Schwann cells and axons in the absence of laminin alpha4. J Neurosci. 2005 Apr 6;25(14):3692-700.

Mats I. Ekstrand, Mügen Terzioglu, Dagmar Galter, Shunwei Zhu, Christoph Hofstetter, Eva Lindqvist, **Sebastian Thams**, Anita Bergstrand, Fredrik Sterky Hansson, Aleksandra Trifunovic, Barry Hoffer, Staffan Cullheim, Abdul H. Mohammed, Lars Olson and Nils-Göran Larsson. Progressive parkinsonism in mice with respiratory-chain-deficient dopamine neurons. Proc Natl Acad Sci U S A. 2007 Jan 23;104(4):1325-30.

Pierre Rotzius, Oliver Soehnlein, Ellinor Kenne, Lennart Lindbom, Kristofer Nyström, **Sebastian Thams** and Einar E. Eriksson. ApoE(-/-)/lysozyme M(EGFP/EGFP) mice as a versatile model to study monocyte and neutrophil trafficking in atherosclerosis. Atherosclerosis. 2009 Jan;202(1):111-8.

Johan Zelano, Alexander Berg, **Sebastian Thams**, Nils Hailer and Staffan Cullheim. SynCAM1 expression correlates to restoration of central synapses on spinal motoneurons after two different models of peripheral nerve injury. J Comp Neurol. 2009 Aug 6;517(5):670-682.

CONTENTS

LIST OF ABBREVIATIONS

ACh	Acetyl Choline
AChR	Acetyl Choline receptor
α-btx	α-bungarotoxin
β_2m	β_2-microglobulin
BBB	Blood-brain-barrier
ChAT	Choline acetyl transferase
CNS	Central nervous system
CL	Contralateral
CTL	Cytotoxic T-lymphocyte
dpo	Days postoperatively
ECM	Extracellular matrix
FACS	Flourescence-activated cell sorting
GM	Gastrocnemius muscle
IFN-γ	Interferon-γ
IHC	Immunohistochemistry
IL	Ipsilateral
IR	Immunoreactivity
MAP-2	Microtubule associated protein 2
MHC	Major histocompatibility complex
MUSK	Muscle specific kinase
NK-cell	Natural Killer cell
P	Postnatal day
PNS	Peripheral nervous system
SC	Schwann cell
SNC	Sciatic nerve crush
SNR	Sciatic nerve resection
SNT	Sciatic nerve transection
Syph	Synaptophysin
TCR	T-cell receptor
TfR	Transferrin receptor
TSC	Terminal Schwann cell
VAChT	Vesicular acetyl choline transporter
WT	Wild-type

1 INTRODUCTION

1.1 THE ANATOMY OF THE SPINAL MOTONEURON

The spinal motoneurons control the principal bodily motors, namely the skeletal muscles. These large neurons extend their long processes, called motor axons, over distances that can measure over 20 000 times the diameter of the soma. These motor axons, which span across the interface between the central (CNS) and the peripheral nervous system (PNS), relay activating electrical impulses to their targets muscles (Fig. 1).

Because of its asymmetric morphology, the different parts of the motoneuron are exposed to considerably different types of extracellular compartments. Firstly, the motoneuron soma (cell body) and dendrites (short processes) reside in the ventral grey matter of the spinal cord surrounded by γ-motoneurons, interneurons and different classes of glial cells, such as oligodendrocytes, astrocytes and microglia. The central part of the motor axon is contained within the white matter where it is ensheathed by myelinating oligodendrocytes. Secondly, the more distal part of the axon exits the CNS through slits in the meninges, thereby crossing the blood-brain-barrier (BBB), to reach the PNS where Schwann cells (SCs) provide the myelination. This is a transition between a central extra cellular milieu, under normal conditions devoid of extra cellular matrix (ECM), to a peripheral milieu where Schwann cells provide ECM in the form of a basal lamina. Thirdly, the peripheral part of the motor axon is relayed in the spinal and peripheral nerves to the skeletal muscles, where the motor terminals form neuromuscular junctions on individual muscle fibres.

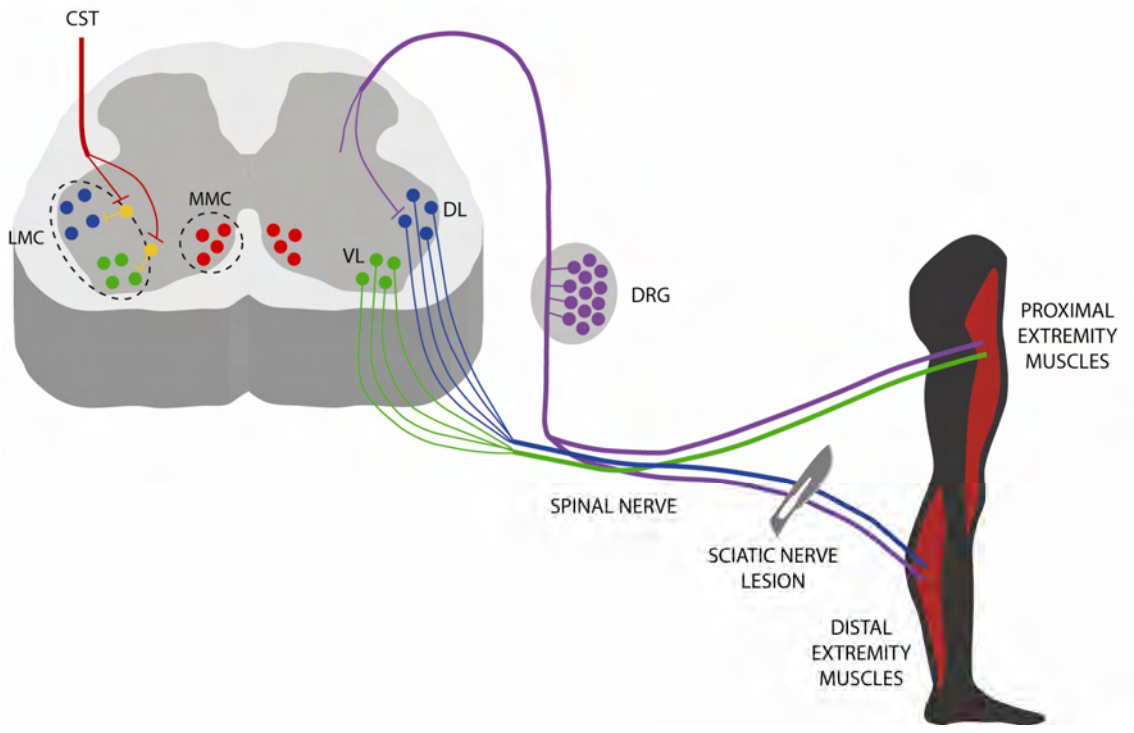

Figure 1: The motoneuron circuitry. A schematic picture showing the two principal motor columns in the spinal cord and their principal trajectories. The medial motor column (MMC) and the lateral motor

column (LMC) are denoted by dashed circles. The ventrolateral (VL) and the dorsolateral (DL) motor pools within the LMC are displayed in green and blue respectively. The lateral corticospinal tract (CST) is displayed in dark red, interneurons are displayed in yellow and dorsal rot ganglion (DRG) neurons are displayed in purple. The location for the sciatic nerve lesion used in this thesis is marked in the picture.

1.2 MOTONEURON CONNECTIVITY

The motoneuron soma and its elaborate dendritic tree are covered by up to 10 000 presynaptic terminals (Ulfhake and Cullheim, 1988), which give rise to excitatory and inhibitory synapses using various neurotransmitters (Fig. 2). A majority of the synaptic input on the motoneuron soma and proximal dendrites is inhibitory and the sources of the terminals are numerous, including local interneurons, primary sensory afferents and descending motor tracts (Fig. 1). The establishment of the motoneuron synaptic input is a largely unknown selective process, which is likely to depend on the guidance by soluble and surface bound cues, neuronal activity and molecular target recognition on soma and dendrites (Glover, 2000; Chakrabarty et al., 2009). The motoneuron is transiently hyperinnervated during development, meaning that the soma and dendrites are contacted by superfluous presynaptic terminals. The refined adult connectivity pattern is obtained through a synaptic elimination process, where inappropriate inputs are retracted (Ronnevi and Conradi, 1974; Conradi and Ronnevi, 1975). How the synaptic elimination process is mediated and which cell types are involved remains unknown. Both the motoneuron itself and adjacent glial cells are believed to actively partake in this process.

The nature of the motoneuron inputs is modulating in its character, indirectly affecting muscle fiber activity. The primary spinal motoneuron output is mediated by acetyl choline (ACh) at the motor endplate.

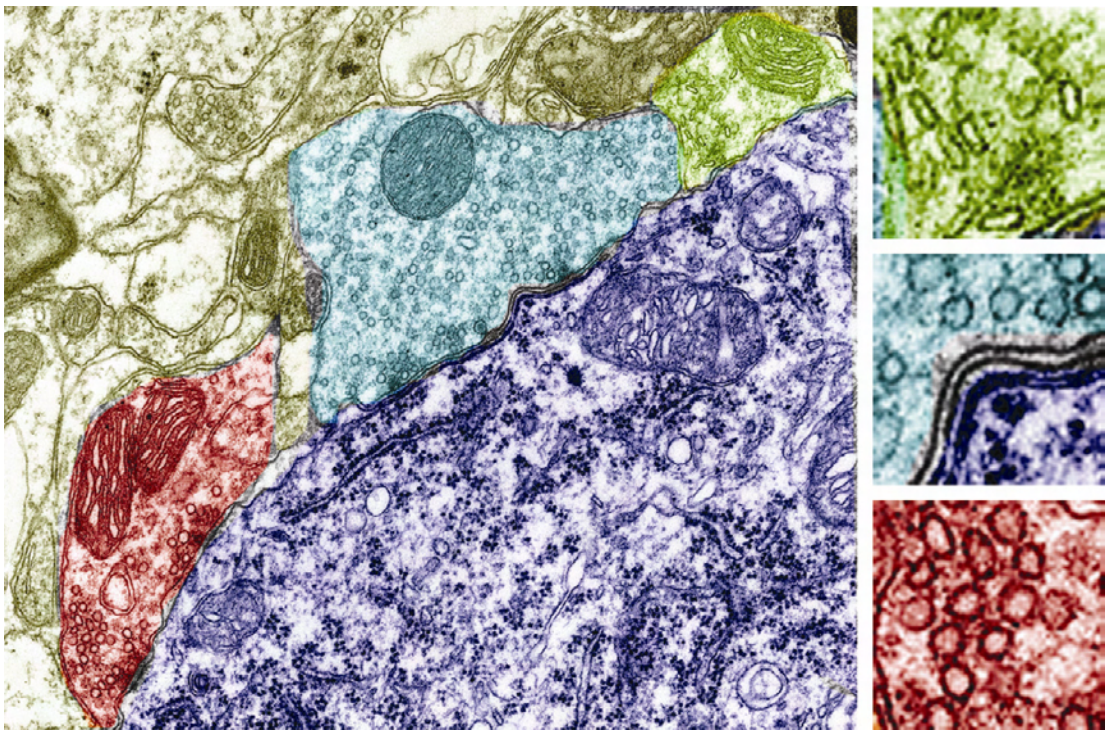

Figure 2: Presynaptic terminals on the motoneuron soma. A pseudocoloured electron micrograph showing the three principal types of synaptic input on the surface of a spinal motoneuron (purple). The S-type terminal (red) is glutamatergic and contains spherical vesicles (bottom right panel). The C-type terminal (turquoise) is cholinergic and contains spherical vesicles. It is characterized by a three layered subsynaptic cistern (middle right panel). The F-type terminal (light green) is GABAergic/glycinerig and contains flat vesicles (top right panel). Original photograph acquired by Alexandre LR Oliveira, currently at State University of Campinas, modified from Fig. 14.2 in the book 'The Sticky Synapse' (1st Edt., 2009, Springer/Kluwer Academic Publishers, Chp 14 by Thams and Cullheim) with kind permission from Springer Science and Business Media.

At the neuromuscular junction (NMJ), a cholinergic presynaptic motor terminal contacts a specialized postsynaptic region on the muscle fibre, termed the motor endplate (Sanes and Lichtman, 1999). The presynaptic terminal is covered and stabilized at the motor endplate by terminal Schwann cells (TSCs), thereby forming a tripartite organization (Gan and Lichtman, 1998; Sanes and Lichtman, 1999; Kim and Burden, 2008). The endplate contains postsynaptic acetyl choline receptors (AChRs) located in membrane invaginations on the muscle fibre. Upon activation, the AChRs give rise to local depolarization that spreads to the entire fibre. Importantly, an activation of the motor endplate always gives rise to a postsynaptic depolarization with subsequent contraction of the fibre.

During development, muscle fibres are transiently hyperinnervated, i.e. one fibre is contacted by several motoneurons and a single fibre can thus have multiple NMJs. In normal adult skeletal muscles, there is only one NMJ per muscle fiber (Gan and Lichtman, 1998; Sanes and Lichtman, 1999; Kim and Burden, 2008). In resemblance to the central connectivity pattern, the mature peripheral muscle synapse organization is also achieved through a developmental refinement process, where redundant synapses are removed on the basis of neuronal and muscle fibre activity. Due to the large size of the NMJ and the good accessibility both *in vivo* and *ex vivo*, it has been possible to study the actual elimination process in real time (Song et al., 2008). By doing so, it has been shown that SCs actively participate in the retraction of presynaptic motor terminals, possibly by means of engulfment (Bishop et al., 2004; Song et al., 2008).

1.3 SOMATOTOPIC MOTONEURON ORGANIZATION IN THE SPINAL CORD

Similar to the primary motor cortex, the spinal motoneurons are somatotopically arranged within the ventral horn. Spinal motoneurons have been divided into strictly defined subclasses by linking the expression of transcription factors to motoneuron trajectories (Tanabe and Jessell, 1996). This section will, however, only give a simplified description aiming at providing sufficient information for this thesis.

The spinal motoneurons are arranged in longitudinal columns (Maden, 2006; Dasen et al., 2008). Along the entire spinal cord, there is a medial motor column (MMC) innervating trunk and body wall musculature. The motoneurons supplying the extremities are conglomerated at the cervical (upper limbs) and lumbar (lower limbs)

intumescences of the spinal cord, where they form a lateral motor column (LMC). Within the LMC, individual motoneurons are arranged so that motoneurons with distal trajectories are located dorsolaterally to motoneurons with a more proximal trajectory (Maden, 2006; Dasen et al., 2008). In this thesis, the LMC is further subdivided into a ventrolateral (VL) motor pool supplying proximal limb muscles and dorsolateral (DL) motor pool supplying distal limb muscles (Fig. 1).

1.4 MOTONEURON AXOTOMY

The term axotomy refers to the transection of the axon. This phenomenon is seen e.g. in relation to a traumatic injury or neurological disease. Post-axotomy, the motoneuron cell body goes through a series of changes as a response to the injury. These changes are aimed at restoring the function of the injured neuron and regenerating the axon in order to reinnervate the target structure, but an axotomy could ultimately lead to cell death. The principal neuronal changes seen after axotomy have been summarized below.

1) **Chromatolytic reaction.** This term refers to morphological changes including retraction of dendrites, misplacement of the nucleus and disassembly of the Nissl substance (Ross et al., 2003).
2) **Gene expression.** The chromatolytic reaction is paralleled by metabolic changes in the motoneuron, in which the genetic profile of the neuron is switched from a ‘transmission mode’ to a ‘survival mode’. Genes associated with neurotransmission is down-regulated and genes involved in regeneration are up-regulated (Davidoff and Schulze, 1988; Arvidsson et al., 1990; Piehl et al., 1991; Hammarberg et al., 1998; Piehl et al., 1998; Hammarberg et al., 2000a).
3) **Synaptic plasticity.** Synaptic terminals are retracted in a process often referred to as ‘synaptic stripping’ (Blinzinger and Kreutzberg, 1968; Brannstrom and Kellerth, 1998), in which the motoneuron soma and dendrites lose around half of their inputs. Excitatory terminals are removed to a higher degree than inhibitory ones (Linda et al., 2000; Oliveira et al., 2004). One explanation for this selectivity in synaptic elimination could be that the motoneuron seeks to minimize excitation, which could propagate further harm through excitotoxicity. Prolonged muscle denervation deprives the motoneuron of neurotrophic support from its innervation target and increases the risk for cell death. If muscle innervation is reestablished, the motoneuron gradually restore its synaptic input (Zelano et al., 2009).
4) **Reactive gliosis.** By largely unknown activation mechanisms, a substantial central gliosis is seen shortly after a peripheral axotomy (Blinzinger and Kreutzberg, 1968). Both microglia and astrocytes show pronounced changes in response to the injury, but the purpose for their activation is paradoxical.

1.5 'IMMUNE MOLECULES' IN NEURONS

1.5.1 Immune privilege

For many years, the CNS was perceived as an 'immune privileged' site due to: the low or absent expression of MHC class I molecules in adult neurons; the lack of rejection of foreign tissue transplanted into the CNS; the restricted migration of most immune cells across the BBB and the high local expression of immunosuppressive soluble factors. An argument that further strengthened the notion of CNS immune privilege due to MHC class I paucity was the finding that neuronal MHC class I immunoreactivity could not even be detected in mice over-expressing interferon (IFN)-γ under an astrocytic promoter (Horwitz et al., 1999). IFN- γ is a powerful inducer of MHC class I expression, therefore high astrocyte-driven secretion of this cytokine is expected to induce neuronal MHC class I expression. Moreover, neurons appeared to be particularly vulnerable to viral infections that required antigen presentation (Joly et al., 1991; Joly and Oldstone, 1992). However, along with accumulating publications showing that neurons in fact can and do express MHC class I proteins under certain conditions; a new concept challenging the traditional view is now emerging. This will be further elucidated in later sections.

1.5.2 Common molecules

The nervous system and the immune system display common features when considering cell to cell interactions through synaptic complexes (Fig. 3), expression of surface molecules and soluble factors (Becher et al., 1998; Darnell, 1998; Dustin and Colman, 2002; Suzuki et al., 2007). Common key molecules have been identified, which can convey interactions between the two systems or mediate separate functions restricted to each system (Tracey, 2002; Stevens et al., 2007; Suzuki et al., 2007; Savarin and Bergmann, 2008; Schwartz and Ziv, 2008). Table 1 shows examples of molecules that are expressed and used independently by both systems.

Table 1. Examples of molecules involved in both immune processes and neuronal plasticity.

Molecule	Immune functions	Neuronal functions	References
MHC class I molecules	- Adaptive immunity: antigen presentation, CTL activation. - Innate immunity: inhibition of NK-cells.	- Synaptic plasticity during development and after nerve lesion. - Surface expression of receptors in vomeronasal sensory neurons.	(Moretta et al., 1992; Germain, 1994; Kagi et al., 1996; Huh et al., 2000; Loconto et al., 2003; Oliveira et al., 2004; Ishii and Mombaerts, 2008)
PIR-B	Immune homeostasis	- Regulation of ocular dominance plasticity. - Myelin mediated inhibition of axon growth.	(Ujike et al., 2002; Nakamura et al., 2004; Syken et al., 2006; Atwal et al., 2008)
Ly49 receptors	Innate immunity: regulation of NK-cell mediated cytotoxicity.	Regulation of neurite branching and synapse formation.	(Lanier, 1998; Zohar et al., 2008)

Molecule	Immune functions	Neuronal functions	References
Complement (C1q, C3)	Innate immunity: chemotaxis, opsonisation, cytotoxicity and removal of immune complexes.	Synaptic refinement in the developing retinogeniculate system.	(Medzhitov and Janeway, 2000; Stevens et al., 2007; Ip et al., 2009)
CD44	- Lymphocyte adhesion. - Lymphocyte mediated anti-tumoural activity.	- Axon-Schwann cell interactions. - Regulation of axon growth.	(Sherman et al., 2000; Zhang et al., 2007; Mrass et al., 2008)
Thrombospondin	Immune modulation: ocular immune privilege.	Induces synaptogenesis.	(Masli et al., 2002; Streilein, 2003; Christopherson et al., 2005; Eroglu et al., 2009)
Semaphorins	Immune regulation: - Inhibits immune cell migration. - T-lymphocyte activation	Axon guidance: axon repulsion, growth cone collapse.	(He and Tessier-Lavigne, 1997; Kikutani and Kumanogoh, 2003; Suzuki et al., 2007)
Neuropilins	Immune regulation: - Inhibits immune cell migration. - T-lymphocyte activation	Axon guidance: axon repulsion, growth cone collapse.	(He and Tessier-Lavigne, 1997; Takahashi et al., 1998; Kikutani and Kumanogoh, 2003)
Plexins	Immune regulation: - Inhibits immune cell migration. - T-lymphocyte activation	Axon guidance: axon repulsion, growth cone collapse.	(Winberg et al., 1998; Kikutani and Kumanogoh, 2003; Suzuki et al., 2007)
Fas/Fas ligand (FasL)	- CTL mediated cytotoxicity. - Immune privilege in CNS and eye.	- Programmed embryonic neuronal cell death. - Immune privilege in CNS.	(Becher et al., 1998; Raoul et al., 1999; Raoul et al., 2000)
Toll-like receptors	Innate immunity: detection of and immune response towards conserved pathogen associated molecular patterns.	- Neurogenesis - Axon guidance - Programmed cell death	(Miller et al., 2005; Ma et al., 2006; Cameron et al., 2007; Larsen et al., 2007; Rolls et al., 2007; Crozat et al., 2009)
Integrins	- Leukocyte adhesion and migration. - Adhesion at the immunological synapse	- Axon growth: interactions with ECM. - Synaptic plasticity	(Werr et al., 1998; Chavis and Westbrook, 2001; Suzuki et al., 2007; Cingolani et al., 2008; Plantman et al., 2008; Liu et al., 2009)

In the CNS, microglia and peripheral immune cells interact with neurons and macroglia through immunological surface molecules and cytokines (Neumann, 2001; Savarin and Bergmann, 2008). This type of neuroimmune interplay is important in the compromised CNS e.g. during neurotropic viral infections and inflammatory disease, such as multiple sclerosis (Piehl and Lidman, 2001; Griffin, 2003; Carson et al., 2006; Rebenko-Moll et al., 2006; Savarin and Bergmann, 2008). Additionally, there are indications for a cross-talk between the immune system and the CNS. For instance, normal adaptive immune function appears to be required for neurogenesis and differentiation during activity-induced plasticity in the hippocampus (Ziv et al., 2006). Similarly, normal innate immune function conveyed by microglia in the developing CNS has been linked to synaptic maturation and developmental apoptosis of neurons in the hippocampus (Roumier et al., 2004; Roumier et al., 2008; Wakselman et al., 2008).

In addition to the involvement of immunological molecules in neuroimmune interactions there are also indications for roles in neuron-neuronal and neuron-glial communication (Huh et al., 2000; Oliveira et al., 2004; Syken et al., 2006; Bessis et al., 2007; Stevens et al., 2007), which may be independent of immune function. Studies of neuronally expressed immune recognition molecules such as the MHC class I family, the classical complement cascade and Toll-like receptors (Huh et al., 2000; Oliveira et al., 2004; Goddard et al., 2007a; Larsen et al., 2007; Stevens et al., 2007), indicate functions related to synaptic plasticity, modulation of neuronal function and regeneration (Corriveau et al., 1998; Huh et al., 2000; Loconto et al., 2003; Oliveira et al., 2004; Barco et al., 2005; Olson et al., 2006; Goddard et al., 2007a).

1.5.3 MHC class I proteins

The Major Histocompatibility Complex (MHC) is a conserved genomic region in jawed vertebrates (Flajnik and Kasahara, 2001), which contains structurally related genes and encode widely-expressed cell surface molecules, such as the MHC class I proteins. Classical MHC class I α-polypeptides (class Ia), which are transmembrane proteins encoded by a few highly polymorphic genes, associate with a β_2-microglobulin (β_2m) polypeptide and contain unique peptide binding clefts that can bind 8-10 amino acid peptides. By presenting such peptides derived from intracellularly synthesized proteins, MHC class I molecules provide a continuous sampling of the intracellular protein synthesis for scrutiny by T-lymphocytes, allowing prompt activation and development of effector cell responses when nonself peptides are presented. Non-classical MHC class I α-polypeptides (class Ib) are encoded by a number of oligomorphic genes, some of which are expressed independently of β_2m or a peptide fragment or both. The number of non-classical MHC class I genes vary substantially between species (Niedermann et al., 1995; Niedermann et al., 1997).

MHC class I molecules are assembled in the endoplasmic reticulum (ER). Correct folding and surface expression of the class Ia molecules is dependent on the association with β_2m and loading of peptide. Generally, peptides that bind MHC class I are derived from the cytoplasm and transferred into the ER by the transporter associated with antigen processing (TAP, consisting of the TAP1 and TAP2 subunits) (Janeway, 2001). In the absence of β_2m, MHC class Ia molecules are trapped in the ER. Moreover, in the absence of TAP, MHC class I molecules are unstable and only a fraction is transported to the cell surface (Ljunggren et al., 1990). Peptides which are

presented by MHC class I molecules are generated from cytoplasmic proteins degraded by the proteasome complex. Thus, the mature MHC class I molecules on the cell surface present a peptide repertoire, which reflects the protein metabolism of the cell. Importantly, MHC class I molecules are expressed by all nucleated cells in jawed vertebrates (Flajnik and Kasahara, 2001).

The function of MHC class I molecules has been extensively studied in the adaptive immune system, where they facilitate thymus-derived cytotoxic T-lymphocyte (CTL) surveillance of tissues for intracellular infections and malignant transformations. CTLs carry clone specific T-cell receptors (TCRs), generated through the somatic recombination of genes, which are specific for unique MHC class I-peptide combinations. MHC class I proteins interact with TCRs through a transient binding state. At the cellular level, this is often referred to as the immunological synapse (Fig. 2). In the thymus, developing thymus derived lymphocytes (T-lymphocytes) that react with MHC class I molecules presenting endogenous peptides are eliminated (negative selection), whereas T-lymphocytes with a weak affinity for MHC class I molecules are positively selected for survival. In this manner, a repertoire of T-lymphocytes with the ability to recognize MHC class I molecules presenting foreign peptides, such as those derived from intracellular pathogens, is generated (Germain, 1994).

Complementing CTL-mediated immunity, natural killer (NK) cells can eliminate cells with down-regulated surface expression of the MHC class I (Moretta et al., 1992). This subset of lymphocytes conveys innate immune functions relying on rapidly evolving germline encoded receptors that bind MHC class I molecules more or less independently of peptide presentation (Zinkernagel and Doherty, 1974; Karre et al., 1986; Ljunggren and Karre, 1990; Bryceson et al., 2006). The NK-cell receptors for MHC class I molecules can be of an inhibitory or activating type. NK-cells can also identify aberrant cells on the basis of expression of stress induced molecules, e.g. the diverse ligands of the activating NK cell receptor NKG2D (Lodoen and Lanier, 2006).

Some MHC class I inhibitory receptors, e.g. PIR-B and LIR receptors, are expressed on a variety of leukocytes within the adaptive and innate immune system. The role of these receptors in the immune response is poorly understood (Ujike et al., 2002; Nakamura et al., 2004).

1.5.4 Neuronal expression and functions of MHC class I

MHC class I immunoreactivity is sparsely detected *in vivo* in the intact adult CNS and mRNA for different MHC class I genes are only found at low to moderate levels in specific neuronal subpopulations. However, mRNA is transiently increased during development, neurotrophic infections, after treatment with IFN-γ and after axotomy. The underlying mechanism for the down-regulation of MHC class I genes is unknown, but a recent publication suggested involvement of epigenetic mechanism such as DNA methylation (Miralves et al., 2007). This hypothesis, however, is of speculative nature and needs to be confirmed.

An essential question regarding neuronal MHC class I expression is whether neurons, like other somatic cells, possess the ability to generate innate and immunogenic peptides. This ability is a prerequisite for interactions with immune cells. Studies of

infections with neurotropic viruses have shed some light on this matter. Whereas some neurotropic viruses, e.g., the herpes virus family, cause a down-regulation of MHC class I in neurons in order to escape immune-mediated clearance, others result in a strong up-regulation of MHC class I, β_2m, TAP1 and TAP2 at the mRNA or protein level (Bilzer and Stitz, 1994; Kimura and Griffin, 2000). Several of the viruses in the second category generate an adaptive CTL-mediated immune response, which results in a partial or complete clearance of the virus. Examples of neurotropic virus that trigger a functional immune response are: Lymphocytic Choriomengingitis Virus (LCMV), Theiler's Mouse Encephalitis Virus (TMEV), Borna Disease Virus, Neuroadapted Sinbis Virus, Rabies Virus and Mouse Hepatitis Virus (Griffin, 2003). CTL-dependent viral clearance of neurotropic infections seems to be mediated either directly (Bilzer and Stitz, 1994; Mendez-Fernandez et al., 2003) through MHC class I-mediated antigen presentation or indirectly, e.g., by the induction of anti-viral Interferons (i.e. α, β and γ) (Giuliani et al., 2003; Rodriguez et al., 2003).

As discussed in the previous section, neuronal MHC class I molecules appear to also participate in processes that are not directly related to immunity. The constitutive expression of different MHC class Ia and Ib mRNAs is restricted to certain brain regions or neuronal subpopulations (Corriveau et al., 1998; Lidman et al., 1999; Linda et al., 1999; Loconto et al., 2003). Furthermore, neuronal MHC class I expression is regulated by neuronal activity and has been linked to activity-dependent neuronal plasticity (Neumann et al., 1997; Corriveau et al., 1998). Its specific regional expression (Boulanger and Shatz, 2004) implies that the role for MHC class I proteins in neurons is not purely immunological as this would require a more general expression.

1.5.4.1 MHC class I in neuronal plasticity

Around a decade ago, several publications reported expression of MHC class I proteins in neuronal subpopulations in the absence of obvious immune system involvement (Maehlen et al., 1989; Corriveau et al., 1998; Linda et al., 1998; Lidman et al., 1999; Linda et al., 1999). In these previous publications we and others showed that MHC class I and β_2m mRNAs were strongly up-regulated by spinal motoneurons in response to axonal lesion, thereby suggesting its involvement in the post traumatic response of these neurons. The function of MHC class I proteins in 'non-immune' contexts remained virtually unknown for another couple of years until a publication by Shatz and co-workers suggested a postsynaptic role for MHC class I proteins in the refinement of retinogeniculate projections during development. In this way, MHC class I molecules are proposed to act also at the neuronal synapse (Huh et al., 2000) (Fig. 3), where they could mediate stabilization or weakening of synaptic contacts in an activity-dependent manner.

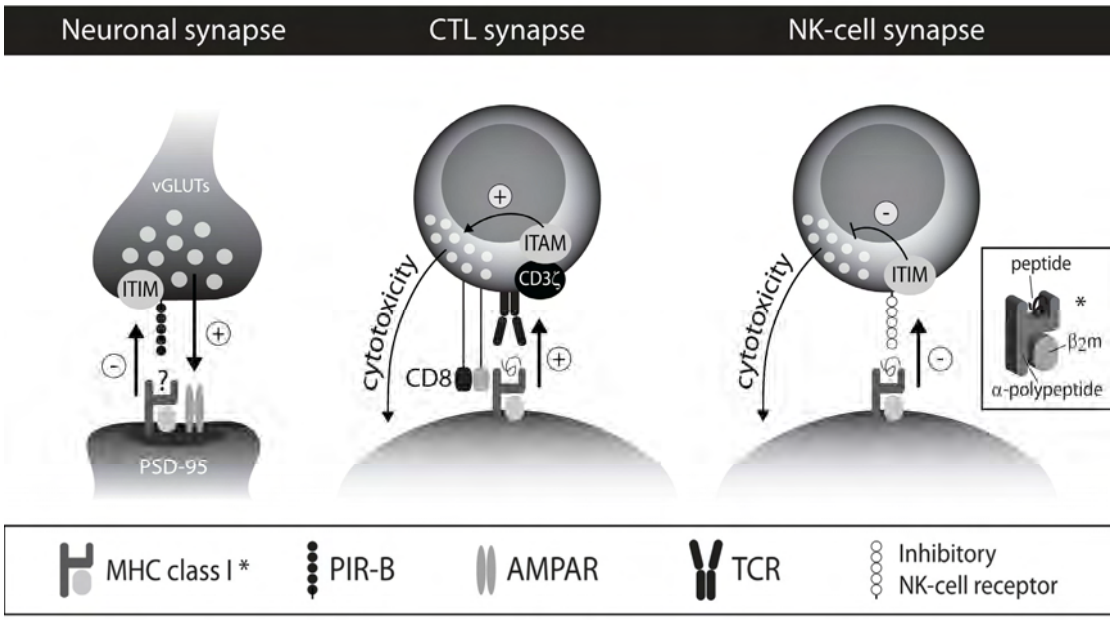

Figure 3: Different types of MHC class I dependent synapses. A simplified schematic figure showing MHC class I dependent synapses in the nervous system and the immune system. **(Left)** An excitatory presynaptic terminal (top) is depicted in connection to a dendritic spine (bottom). Normal synaptic transmission is conveyed through vesicular glutamate release, which activates e.g. postsynaptic AMPA-receptors (AMPAR), leading to excitation (+) in the postsynaptic neuron. Neuronal MHC class I molecules are co-localized *in vitro* with postsynaptic density 95 (PSD-95) in association with glutamatergic presynaptic terminals (vGLUTs = vesicular glutamate transporters). The first MHC class I receptor reported in neuronal cells was PIR-B, which is localized to presynaptic terminals. PIR-B has immune tyrosine inhibitory motifs (ITIMs) and thus conveys an inhibitory signal (-) in the opposite direction across the synaptic cleft. It may possibly lead to an inhibition or the retraction of the presynaptic terminal. **(Middle)** At the cytotoxic T-lymphocyte (CTL) synapse, MHC class I molecules on target cells present a peptide fragment to a CTL, leading to an activating signal (+) if the TCR recognized the peptide fragment as foreign. The CD8 molecule serves as a co-receptor, which binds to the MHC class I α-polypeptide, enabling an MHC class I-TCR interaction. The TCR is associated with the CD3 complex, which contains an immune tyrosine activating-motif (ITAM) on the CD3ζ subunit. Upon activation, the CTL kills the target cell, e.g., by releasing cytotoxic granulae, thus mediating an adaptive immune response. **(Right)** At the natural killer (NK) cell synapse, MHC class I molecules on the target cell interacts with a display of NK-cell receptors, resulting in an inhibitory response. If MHC class I molecules are expressed on the cell surface to a normal extent, an inhibitory signal is conveyed across the synapse through ITIM-associated NK-cell receptors, thereby preventing degranulation. If however, the levels of MHC class I molecules are decreased on the cell surface, the NK-cell will release its cytotoxic granules and kill the target cell, thus mediating innate immunity. Reproduced from Fig. 14.3 in the book 'The Sticky Synapse' (1st Edt., 2009, Springer/Kluwer Academic Publishers, Chp 14 by Thams and Cullheim) with kind permission from Springer Science and Business Media.

1.5.5 The complement cascade

Complement proteins are circulating immune recognition and effector molecules that participate in innate immunity. A detailed description of the complement cascade is beyond the scope of this thesis, and the interested reader is referred to review articles

on the subject (Medzhitov and Janeway, 2000; Ip et al., 2009). Briefly, the complement cascade can be initiated through three principal pathways. The 'classical pathway' is initated when C1q binds to antibodies bound to antigens on a bacterial surfaces (Janeway, 2001). The 'mannan-binding lectin pathway' is activated by a circulating lectin, which binds to conserved carbohydrate epitopes on bacteria or viruses. Finally, in the 'alternative pathway' C3b is directly bound to pathogen surfaces. All three pathways converge on the enzymatic formation of C3, which is the key molecule that covently binds to pathogens. Downstream of C3 different effector molecules that mediate chemotaxis of immune cells, opsonisation, clearance of immune complexes and cytolysis are generated.

From a nerve injury point of view, complement molecules participate in the clearance of debris and axonal remnants after a peripheral nerve lesion and their function could therefore affect the regeneration process (Bruck and Friede, 1990; Dailey et al., 1998; Ramaglia et al., 2007). The role of complement proteins have been extensively studied in the immune system and this thesis will mainly focus on the newly studied 'non-immune' roles in neuronal plasticity.

C1q, one of the initiating proteins in the cascade, has been studied in the nervous system and is shown to be expressed by retinal ganglion cells when co-cultured with astrocytes and *in vivo* during retinal development (Stevens et al., 2007). The neuronal expression of these proteins was linked to synaptic refinement of retinogeniculate projections. The study by Stevens et al. 2000, provides certain histological indications that C1q and C3, the key molecule in the cascade, can tag immature synapses and thereby label them for removal by adjacent glial cells, but this remains to be fully validated on the ultrastructural level. Nonetheless, C1q and C3 null mutant mice ($C1q^{-/-}$ and $C3^{-/-}$) are convincingly shown to retain an unrefined innervation pattern, with a higher number of synaptic inputs on LGN neurons than wild-type (WT) mice, after the period when normal synaptic pruning can be expected to have occurred (Stevens et al., 2007). Moreover, the synaptic inputs that remain on the mutant LGN neurons display immature electrophysiological properties. Although much remains to be investigated regarding the role and site of action of complement in synaptic refinement, there are clearly indications that these proteins play an important role in synaptic elimination, independently of the immune system.

1.6 TARGET RECOGNITION IN THE NERVOUS SYSTEM

In difference to the well characterized accuracy of many cellular interactions in the immune system, the ultimate target recognition process for outgrowing nerve processes remains elusive. One way for growing axons to reach their targets is to use pre-existing ones, so called pioneer axons, as guides. Another important factor is guidance by different molecular cues in the local environment. Axon growth promoting cues include various ECM molecules (e.g. laminins, fibronectin and collagen) and adhesion molecules (e.g. cadherins, N-CAM and catenins) (Song and Poo, 2001; Kiryushko et al., 2004). In addition, the target muscles secrete soluble neurotrophic factors that attract motor axons (Song and Poo, 2001). Examples of axon repelling or growth inhibiting molecules are: semaphorins, ephrins, and myelin associated molecules, to

mention a few (Tessier-Lavigne and Goodman, 1996; Song and Poo, 2001; Guan and Rao, 2003; Skaper, 2005). When the motor axons reach the muscles, they form muscle synapses at predefined sites expressing a molecular pre-pattern consisting of e.g. AChRs and muscle specific kinase (MUSK) (Sanes and Lichtman, 1999; Witzemann, 2006). This is a very precise process where different muscle segments are innervated by motoneurons at specific cranial to caudal location (Chadaram et al., 2007; Dasen et al., 2008). Synapse formation is induced by the release of agrin from motor terminals, which interacts with MUSK. Other molecules involved in the synapse assembly include: muscle derived neuregulins, ErBs, rapsyn and AChRs (Sanes and Lichtman, 1999; Arber et al., 2002; Witzemann, 2006). However, it is unknown how an individual motor axon initially locates its exact position to form a synapse. Even though the role of immune recognition molecules is not investigated in motoneuron target recognition, it is tempting to hypothesize that such molecules can be used by neurons for this purpose.

2 AIMS

The aim of this thesis was to study the expression and possible functions of traditional immune molecules in motoneurons with regard to structural plasticity and regeneration.

The specific objectives were:

1. To characterize mRNA and protein expression of MHC class I molecules, complement C1q and C3 in motoneurons and their microenvironment.

2. To investigate possible roles for these molecules in structural plasticity and regeneration of motoneurons following nerve lesion.

3. To search for potential receptor molecules for MHC class I and complement in neurons and glial cells.

4. To formulate hypotheses regarding possible cellular interactions in the spinal cord and PNS that are dependent on or involve neuronally expressed MHC class I molecules or complement proteins.

3 METHODOLOGICAL CONSIDERATIONS

3.1 GENTICALLY MODIFIED ANIMALS USED IN THIS THESIS

The following genetically modified mouse strains on C57 BL/6 background were used in this thesis:

MHC class I-deficient animals:
$\beta_2m^{-/-}$ mice lack all classical and most non-classical MHC class I molecules
$TAP1^{-/-}$ mice lack all classical and a subset of non-classical MHC class I molecules
$TAP1^{-/-}\beta_2m^{-/-}$ mice lack a majority of all MHC class I molecules
$K^{b-/-}$ mice lack half of all classical MHC class I molecules
$D^{b-/-}$ mice lack half of all classical MHC class I molecules
$K^{b-/-}D^{b-/-}$ mice lack all classical MHC class I molecules

Complement-deficient animals:
$C3^{-/-}$ mice lack C3 proteins

To my knowledge, a major concern regarding most current publications (including the ones in this thesis) dealing with neuronal functions of immune molecules, is that these studies have so far only been carried out in global null mutant mice. The influence of a malfunctioning immune system is therefore a relevant confounding factor. There are reports indicating that normal adaptive immune functions are essential for normal development of the nervous system (Ziv et al., 2006). In this thesis, this problem has only briefly been dealt with, when control experiments were carried out in the $RAG1^{-/-}$ mouse strain, which suffers from a severe immunodeficiency (**Paper I**).

As displayed in the list above, there are several null mutants for MHC class I proteins and associated molecules, but none of them abolish the MHC class I expression completely. Hence, it is not possible to study a complete absence of MHC class I molecules using these animals, even though $TAP1^{-/-}\beta_2m^{-/-}$ animals are often used as a pan-null mutant control.

Another issue is that multiple cell types in the CNS express immune molecules and it is therefore difficult to distinguish the precise role of the neuronal expression of these molecules from the e.g. glial expression. The only way to resolve this issue would be to generate conditional null mutants that are genetically engineered to lack expression of a certain immune molecule in a specific cell population.

Finally, the $\beta_2m^{-/-}$ and $TAP1^{-/-}\beta_2m^{-/-}$ mutants also have perturbed functions in organ systems other than the immune or nervous system. For instance, these animals lack functional expression of HFE, a non-classical MHC class I molecule, which associate with β_2m and interacts with the transferrin receptor (TfR) (Salter-Cid et al., 2000; Muckenthaler et al., 2004). The TfR is expressed on cell surfaces and functions as a transmembrane carrier protein for circulating transferrin-iron complexes. The interaction between transferrin and TfR leads to the release of iron into the cytosol. Even though the exact function of HFE molecules in iron metabolism is elusive, a

human mutation in HFE gene leads to a disease called hemochromatosis, which is characterized by tissue iron overload (Salter-Cid et al., 2000; Cardoso et al., 2002; Muckenthaler et al., 2004). β_2m-deficient animals can therefore at an old age be used an experimental model for hemochromatosis. The work in this thesis was therefore carried out in young adult animals. In addition, a prior publication investigating iron overload in $\beta_2m^{-/-}$ mice did not detect any signs of iron acculumlation in the brain (Moos et al., 2000). Moreover, mice lacking classical MHC class I molecules, such as the $K^{b-/-}D^{b-/-}$ strain, also appear to have an iron overload phenotype (Cardoso et al., 2002), suggesting that classical MHC class I molecules also participate in the iron metabolism.

3.2 EXPERIMENTAL NERVE LESION MODELS

Three different types of sciatic nerve lesions have been used in this thesis. These lesions affect motor axons as well as sensory and autonomic axons. We focused on the regeneration of motoneurons since restoration of their function is of particular importance for the functional recovery of an individual after nerve lesion from a clinical perspective.

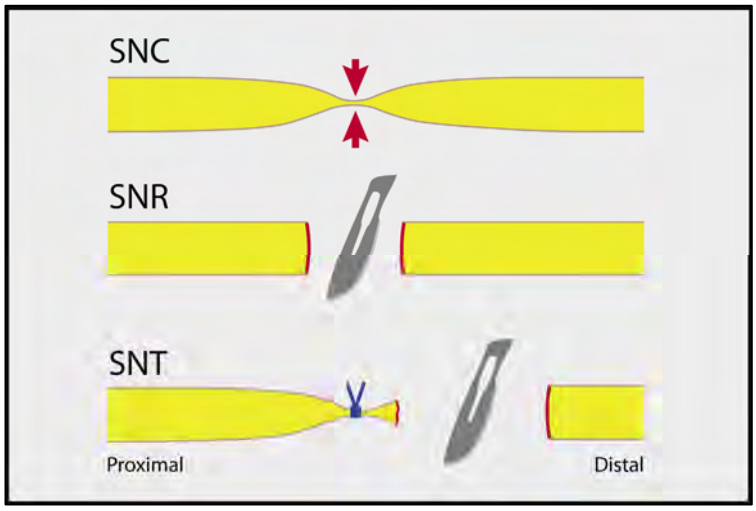

Figure 4: Sciatic nerve lesion models. An illustration of the three different types of sciatic nerve injury used in the thesis.

The **sciatic nerve crush (SNC)** is an experimental model which favors axonal regeneration. The nerve is crushed with a pair of forceps, thereby transecting the axons, but leaving the epi-, peri- and endoneurium more or less intact (Bridge et al., 1994). In this fashion, the severed axons are still contained within the endoneurial tubes providing ECM and proximity to Schwann cells (SCs), which are crucial for supporting and facilitating the axonal regeneration. A sciatic nerve crush affects the motor and sensory functions distal to the knee, resulting in a visible complete paralysis of the affected hind limb. In normal young adult mice, the motoneurons display a robust regenerative capacity and most motor axons reach the hind limb muscles in approximately two weeks after the nerve crush and by three weeks most AChR-clusters are reinnervated. At this time point, a substantial recovery of the hind limb function can be observed. In mice, the crude hind limb muscle function at several weeks after nerve crush is more or less indistinguishable to the uninjured extremity. The total motoneuron cell death is low in this model.

This model offers a possibility to study adult synaptic formation and elimination in motoneurons *in vivo*. Initially after nerve crush, all motor terminals degenerate in a process called *Wallerian degeneration* and all AChRs become denervated. During the regeneration phase, motor terminals successively grow back into the muscle and reestablish connections with the muscle fibres. Due to sprouting of motor terminals, many muscle fibres become hyperinnervated during this phase, i.e. several motoneurons can contact the same muscle fibre and individual muscle fibres can have several NMJs. In resemblance to development, the inappropriate connections are eventually removed during the first and second month after reinnervation.

The **sciatic nerve resection (SNR)** allows limited axonal regeneration. In this model, a segment of the nerve is resected and the stumps are left with a defined gap in between them. A connective tissue bridge connecting the nerve stumps is eventually formed and the axons regenerate across the gap. Muscle denervation is prolonged and the regenerating axons lack guiding structures, thereby resulting in a substantial degree of misdirected axon growth. Muscle recovery of the hind limb is poor or completely absent in normal young adult mice even at 10 weeks after operation. This model stresses the system more than SNC and most likely causes a small to moderate motoneuron death.

The **sciatic nerve transection (SNT)** is aimed at preventing any form of axonal regeneration and the nerve is therefore ligated before a large segment is resected. This model is suitable for studying the central axotomy reaction including motoneuron cell death in the spinal cord in absence of regeneration.

Surgery is always a procedure with experimental variation. This confounder has been dealt with by operating WT and mutant animals at the same occasion and in a random and unbiased order. In addition, the animals were age, gender and weight matched before the procedures.

3.3 RADIOACTIVE *IN SITU* HYBRIDIZATION HISTOCHEMISTRY

One problem with *in situ* hybridization using mRNA probes directed against specific MHC class I α-chains is the great sequence overlap due to resemblance of closely related genes, which was the case for the probes for H2-K^b and H2-D^b (NCBI GeneBank). Three probes with acceptable features in a gene BLAST search were tested on $K^{b-/-}D^{b-/-}$ mice. Only one probe gave a result that was indistinguishable from the background level, even though all of them gave clearly reduced labelling in $K^{b-/-}D^{b-/-}$ mice. A β_2m probe was tested with negative result (i.e. indistinguishable from background) on $\beta_2m^{-/-}$ animals. The spleen was used a positive control.

3.4 IMMUNOHISTOCHEMISTRY AND CONFOCAL MICROSCOPY

Along with the general technical progress, scanning laser confocal microscopy has developed substantially during the last decade. The microscopes now operate at a low laser excitation level, the scanning speed is rapid and the detectors are very sensitive. This has led to a great progress in detecting e.g. small objects at high magnification,

structures at a great tissue depth and detection of weak signals. However, this has simultaneously led to false positive detection since the detector settings are subjected to personal bias. To overcome some of these problems, all MHC class I stainings in this thesis were tried in all available MHC class I null mutants to determine specificity and the background level could be set using the settings achieved in mutants that should lack signal completely.

For measurements of the immunostaining intensity for synaptic or glial markers, general microscopy settings were kept similar and a ratio between the ventrolateral/dorsolateral motor pool or the ipsilateral/contralateral or side of the spinal cord was always calculated to correct for the background levels in each section. All pictures were also acquired blindly with regard to genotype. All secondary antibodies used were incubated on tissue without the presence of primary antibody in order to exclude unspecific secondary staining. In general antibodies raised in mice were carefully tested for unspecific primary or secondary staining before used.

For many classical immune molecules, commercial antibodies suitable for immunohistochemistry (IHC) are lacking, and the ones that are available are often not published or tested carefully for specificity. We therefore made some effort to characterize the MHC class I antibodies used in this thesis. Several different antibodies were tested, but many failed to give immunosignal or gave positive signal also in $TAP1^{-/-}\beta_2m^{-/-}$ animals, which lack a majority of all MHC class I proteins and serve as the only available 'pan-negative' control. In the end, we only found one antibody (ER-HR 52 clone) that gave a consistent staining pattern and that was negative in many of the MHC class I-deficient mice. This antibody was first tested with fluorescence-activated cell sorting (FACS) on tail vein blood from WT and MHC class I-deficient mice and was determined to be mainly specific for the $H2\text{-}D^b$ haplotype. This specificity was confirmed with IHC on the spinal cord and brain stem (see **Paper I**).

For antibodies against the paired immunoglobulin receptor B (PIR-B), no null mutants were available and we therefore had to rely on conventional ways of excluding unspecific staining (e.g. comparison between tissues known to express PIR-B and those that could be expected to negative or low in expression, secondary incubation in the presence and absence of a prior primary antibody).

For complement IHC, we used a C3 and a C1q antibody. The C3 antibody was tested on sections from injured sciatic nerves from $C3^{-/-}$ mice which gave a completely negative result in difference to WT. The C1q antibody was tested using the conventional protocol for excluding non-specific staining.

3.5 ELECTRON MICROSCOPY

To count the number of synaptic terminals on the surface of intact and axotomized motoneurons using electron microscopy is an accurate way of quantifying the synaptic covering of the soma. However, the synaptic terminals present on the soma only account for a minority of the total number of synaptic terminals, since a majority is present in the dendritic tree. Therefore, the synaptic covering of the soma is only a

sample of the total motoneuron covering and it is unknown whether the plasticity of these terminals is representative for the entire motoneuron input. In fact, there are indications that the soma and proximal dendrites lose relatively more synaptic terminals after axotomy compared to the more distal part of dendrites (Brannstrom and Kellerth, 1998). However, it is only possible to make reliable counts of the synaptic covering on the soma and possibly proximal dendrites, since the distal parts of the dendritic tree are discontinuous with the soma in ultra thin sections. In addition, it is known that the more proximally located synaptic terminals have a greater postsynaptic influence on the motoneuron than the more distal ones and may therefore be relevant to study when assessing how a severed motoneuron is affected.

3.6 FUNCTIONAL AND HISTOLOGICAL MUSCLE REINNERVATION AFTER NERVE CRUSH

In **Paper I**, the return of hind limb grip ability was used as crude measure of the time course and quality of muscle reinnervation after a sciatic nerve crush. In order to correct for subjectivity and bias, all behavioural testing was carried out blindly by one main observer. At random occasions different second observers were also present at the scoring in order to confirm or disagree with the results of the first observer.

In parallel with the behavioural testing, the density and distribution of NMJs were estimated in the gastrocnemius muscle (GM). When evaluating the return of grip ability in relation to the histological muscle features, one should consider the fact that the hind limb grip test mainly involves more distal muscles than the GM. The reason for choosing this type of test is its feasibility and is reproducibility even to a relatively untrained scientist. The GM was chosen for histological analysis for its large size, which facilitates reproducible tissue sectioning, and for its well documented features. One may thus anticipate a slight time lag for reinnervation of more distal muscles in the leg compared to the GM.

In the histological analysis, the NMJ density (number of NMJs/ mm^2 muscle area) was calculated using counts obtained in muscle sections. Except for the counted number of NMJs, the density is obviously also relying on the area of the muscle. Since WT muscles carry one NMJ per muscle fibre, the individual cross sectional muscle fibre area is a determinant of the NMJ density. We therefore studied the muscle architecture in GMs from normal WT and MHC class I-deficient mice by measuring the muscle fibre cross sectional areas and arrange them into a frequency histogram. No obvious difference was observed between the two mouse strains. Substantial transient muscle atrophy is seen after nerve crush and we therefore studied the mean muscle fibre cross sectional area at an early and a late time point during reinnervation. No significant difference was seen between the groups.

4 RESULTS AND DISCUSSION

4.1 EXPRESSION OF MHC CLASS I MOLECULES IN AXOTOMIZED MOTONEURONS

In **Paper I** and preliminary results, we detected MHC class I immunostaining for H2-D^b and mRNA for H2-K^b, H2-D^b and H2-T22 in inact spinal motoneurons. The neuropil was weak in signal. At the mRNA level, the signal was increased by several-fold for both H2-K^b/D^b and H2-T22 in axotomized motoneurons. H2-K^b/D^b expression was also strongly increased in glial elements in the neuropil. Strikingly, there was a clear discrepancy between the strongly elevated mRNA levels in axotomized motoneurons and lack of increase in immunosignal between intact and axotomized motoneurons, which was somewhat puzzling. The immunosignal was clearly increased in microglia surrounding the axotomized motoneurons. One possible explanation could be that MHC class I proteins produced in the soma are centrifugally transported into neuronal processes, e.g. in the dendritic tree as proposed by studies *in vitro*. In support of this notion, there are several publications showing or indicating the presence MHC class I molecules in axons and dendrites (Medana et al., 2001; Zhong et al., 2006; Ishii and Mombaerts, 2008; Taylor et al., 2009). However, we failed to detect any clear localization of H2-D^b to motoneuron dendrites and we further investigated whether MHC class I proteins could be detected in the motor axons, as indicated in an *in vitro* experiment in a motoneuron cell-line. Indeed, MHC class I immunoreactivity (IR) was detected in large axons in the intact sciatic nerve and in distal axon stumps in the transected sciatic nerve.

Moreover, we also found a post-axotomy up-regulation of the MHC class Ib mRNA, H2-T22, which was restricted to motoneurons and completely absent in activated glia (Fig. 5). This finding may reflect simultaneous motoneuron expression of multiple MHC class I types acting in different subcellular compartments of the neuron.

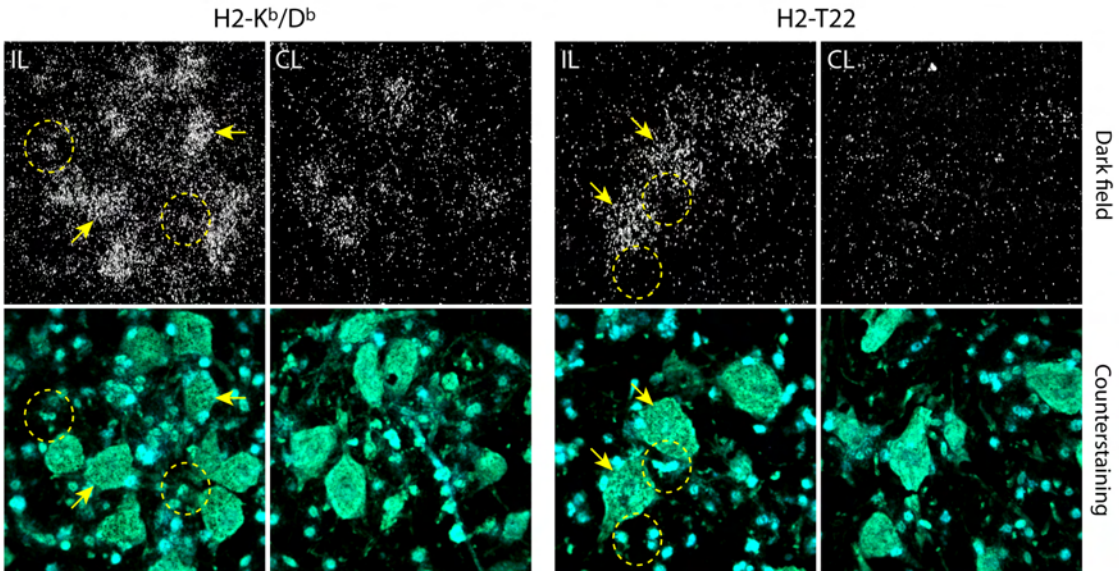

Figure 5. *In situ* hybridization histochemistry for classical and non-classical MHC class I mRNAs. Upper row of panels display dark field images of the ipsilateral (IL) and contralateral (CL) sciatic motor pool hybridized with probes specific for H2-K^b/D^b (classical) or H2-T22 (non-classical).The lower row of panels show vertically corresponding counterstainings with bisbenzimide. Circles denote glial cells and arrow denote motoneurons.

4.2 A ROLE FOR MHC CLASS I FUNCTION AT THE NMJ

In **Paper I**, we found MHC class I IR in a subpopulation of NMJs in both intact and reinnervated hind limb muscles. By examining confocal z-stacks acquired at a short section interval (0.01 μm), we were able to determine the localization of the MHC class I IR to the *presynaptic* side of the NMJ, in comparison to the reports from Shatz and co-workers of a *postsynaptic* role for MHC class I proteins in cultured hippocampal neurons (Goddard et al., 2007b). Although the staining overlapped well with presynaptic markers, such as synaptophysin (syph) and vesicular acetyl choline transferase (VAChT), MHC class I-IR could also simultaneously be present in TSCs. Since only a subpopulation of NMJs, often clustered together, were MHC class I positive; one could speculate that they represented the same motor unit. We were, however, unable to make successful co-stainings for MHC class I and marker for muscle fibre subtypes.

The finding of H2-D^b IR at NMJs led us to examine whether the synaptic organization was altered in $K^{b-/-}D^{b-/-}$ mice. Interestingly, these mice displayed a moderate increase in the density of NMJs in intact hind limb muscles. We then investigated if this phenotype was similarly present in other muscle groups. In whole-mount preparations of the diaphragm muscle from WT mice, the NMJs formed strictly arranged synaptic bands running along the muscle. In $K^{b-/-}D^{b-/-}$ mice, we consistently observed segmental misalignments of the synaptic bands exclusively in the right hemidiaphragm, near the entry zone for the right phrenic nerve. The NMJ density in the right hemidiaphragm from $K^{b-/-}D^{b-/-}$ mice was also higher than in WT mice. Initally, we had no good explanation for this asymmetric and highly localized disturbance. However, by searching the literature we found a publication showing that the diaphragm is developmentally innervated in an asymmetric way (Laskowski et al., 1991). While the left phrenic nerve follows a direct course to the left hemidiaphragm, the right phrenic nerve accompanies the inferior vena cava and therefore reaches its target at a slighty later developmental time point. In addition, the right phrenic nerve grows at a higher speed and gives rise to longer branches than the left phrenic nerve in order to compensate for the longer distance. Altogether, this could be indicative of a developmental disturbance in muscle innervation. We therefore proceeded by studying the postnatal development of the synaptic bands in the diaphragm. In WT mice, we observed a similar asymmetrical, but transient misalignment at postnatal day 0 (P0) and P4 in the synaptic bands, which was corrected for and became less pronounced at P6 and P8 and was not present at P42. In $K^{b-/-}D^{b-/-}$ mice, however, this misalignment was retained into adulthood.

From these results, we conclude that classical MHC class I molecules are important for regulating the synaptic density in the muscles, perhaps by facilitating synaptic

elimination, and for the accuracy of synapse band formation. These results are in line with the reports by Shatz and co-workers from the visual system (Huh et al., 2000).

4.3 $K^{B-/-}D^{B-/-}$ MICE DISPLAY NMJ ABNORMALITIES AND A DELAYED RECOVERY AFTER NERVE CRUSH

The results obtained in intact muscles indicated an impaired elimination of NMJs during development. Accordingly, we hypothesized that such a disturbance was recapitulated in adult injury-induced plasticity, which in many ways resembles developmental processes. In order to test this hypothesis, we performed a time course analysis for the NMJ density in the hind limb muscles of WT and $K^{b-/-}D^{b-/-}$ mice during recovery after SNC (**Paper I**). Both groups showed a similarly elevated density at 14 dpo, indicating that the early hyperinnervation phase occurred to the same extent in the two groups. The WT then displayed a distinct decline in NMJ density, as an effect of synaptic elimination and muscle hypertrophy. The $K^{b-/-}D^{b-/-}$ group remained at a high density at the later time points. This difference could not be expected to result from a difference in muscle atrophy, since no significant difference in muscle fiber cross sectional area was detected at two time points during muscle reinnervation. We interpret this as a sign of a less efficient elimination of NMJs in the $K^{b-/-}D^{b-/-}$ group.

The abnormal NMJ density and the disturbed organization of the synaptic bands in $K^{b-/-}D^{b-/-}$ mice could have at least two possible explanations. Firstly, the absence of presynaptically expressed MHC class I molecules could impair a neuron-muscle interaction, perhaps used to stabilize synaptic contact or recognizing the molecular pre-pattern marking the location for synapse formation. We were unable to experimentally test this alternative. The other possible explanation would concern the third component of the NMJ, namely the TSC. These cells are known to guide regenerating axons to their muscle fibre targets and can remove inappropriate motor terminals and could thus be interesting from a plasticity point of view. We started examining TSC covering at the NMJ in normal muscles and during muscle reinnervation. In intact muscles, fewer TSCs covered and stabilized individual NMJs in $K^{b-/-}D^{b-/-}$ compared to WT mice. During the reinnervation phase, both WT and $K^{b-/-}D^{b-/-}$ TSCs initially proliferated at individual NMJs to a similar degree. The WT group then increased sharply in TSC covering at NMJs at 30 dpo, which is the active remodeling phase. The $K^{b-/-}D^{b-/-}$ mice remained at a similar level compared to earlier time points. This could support the notion of an impaired MHC class I dependent interaction between TSCs and motor axon as an explanation for the altered NMJ dynamics during reinnervation.

At the behavioural level, the crude recovery of muscle function after SNC was estimated with the return of grip strength. In line with this parameter, the $K^{b-/-}D^{b-/-}$ mice showed a different dynamic pattern compared the WTs. The WT mice recovered their grip ability very quickly, while the $K^{b-/-}D^{b-/-}$ mice had substantially prolonged recovery period. While these results cannot directly be ascribed to the histological phenotype, it is not entirely farfetched to consider this possibility. Several papers show that the histological features during muscle reinnervation correlate to functional properties of the muscle (Verdu and Navarro, 1997; Magill et al., 2007). Moreover, it is known that

reinnervated NMJs do not obtain fully mature properties until redundant NMJs have been removed (Magill et al., 2007).

4.4 POTENTIAL MHC CLASS I RECEPTORS

Subsequently, we tried to elucidate possible cellular interactions underlying the abnormal dynamic changes in NMJ density seen in $K^{b-/-}D^{b-/-}$ mice during muscle reinnervation after SNC. If MHC class I molecules truely act at the synaptic level, as proposed for the visual system by Boulanger and Shatz 2004, receptors are likely to be present in the postsynaptic membrane. In this thesis, we were unable to design a screening study for other MHC class I receptors than those previously known from the immune system.

Few MHC class I receptors have been identified in neurons or their target cells so far in the literature. Nevertheless, there are two publications showing expression and neuronal function of PIR-B (Syken et al., 2006) and members of the Ly49 receptor family (Zohar et al., 2008) respectively. For that reason, we screened for these receptors in our model systems (**Paper I**). We did not detect any immunostaining in the spinal cord *in vivo* for either class of receptors. Since we detected a disturbance in TSC proliferation during muscle reinnervation, we hypothesized that these cells could be candidates for expressing MHC class I receptors. In order to broaden the repertoire of antibodies, we used a flow cytometry approach where we dissociated SCs from postnatal sciatic nerves. We screened for several Ly-49 receptors and PIR-A and B. We found expression of PIR-B in a subpopulation of SCs. This was also confirmed with IHC on the adult sciatic nerve. Even though this experiment was purely morphological, it still provides a molecular basis for a MHC class I-related signalling between motor terminals and SCs through PIR-B. We did not detect PIR-B IR at the NMJ and accordingly conclude that outgrowing MHC class I expressing axons interact with SCs and TSC precursors proximal to the NMJ, perhaps in order to receive guidance during reinnervation.

4.5 DISTURBED CENTRAL MOTONEURON PLASTICITY IN ABSENCE OF MHC CLASS I FUNCTION

A publication by Huh et al. 2000 demonstrating MHC class I involvement in developmental CNS plasticity led us to investigate if these molecules were also involved in adult axotomy-induced plasticity. Consequently, we studied the 'synaptic stripping' seen in spinal motoneurons after axotomy in **Paper II**. Based on the findings by Huh et al 2000, we initially hypothesized that animals lacking MHC class I molecules would display a reduced elimination of synapses from the somata of axotomized motoneurons. Somewhat surprisingly, one week after a sciatic nerve transaction, both $\beta_2m^{-/-}$ and $TAP1^{-/-}$ mice showed a ~50% larger reduction in syph-IR in the DL motoneuron pool compared to WT mice. Staining and quantification of the microtubule associated protein 2 (MAP-2), a dendritic marker, did not reveal any difference between the groups regarding dendritic retraction (data not shown). This

would signify a greater loss of synapses from axotomized motoneurons, which was confirmed at the ultrastructural level. Motoneurons in axotomized $\beta_2m^{-/-}$ animals indeed displayed a greater loss of presynaptic terminals from their somata. The aggravated synaptic elimination in the $\beta_2m^{-/-}$ mutants was selective in the sense that inhibitory so called F-type terminals (Fig. 2) were lost to a higher degree compared to WT mice. Furthermore, the normal clustered pattern of the synaptic terminals was lost and the remaining terminals were scattered along the motoneuron membrane, again indicative of disturbed elimination process. This could suggest that the $\beta_2m^{-/-}$ motoneurons were subjected to a shift in excitation versus inhibition, which in turn could lead to excitotoxicity, impaired recovery or even cell death. No signs of accelerated motoneuron death were detected in $\beta_2m^{-/-}$ animals when counting the number of remaining motoneurons at three weeks after axotomy. There was, however, a difference in the number of regenerating axons that were able to cross the gap between the proximal and distal nerve stump after a sciatic nerve resection, where the WT mice displayed a more efficient axonal regeneration than $\beta_2m^{-/-}$ mice.

The apparent contradiction between our result and that of Shatz and co-workers (Huh et al., 2000) is hard to interpret at this point, considering the lack of mechanistic studies. Nonetheless, some efforts to discuss this have been provided below; even though it must be considered highly speculative.

Shatz and co-workers propose a postsynaptic role for MHC class I molecules, where they convey some kind of retrograde retraction or stabilization signal to the presynaptic terminal. Consider the following speculative and fitted hypothesis: MHC class I molecules are induced at recently established electrically ‘weak’ synapses, as previously suggested (Neumann et al., 1997). When expressed at the synapse, the MHC class I molecules convey a retrograde signal to an immature PIR-B expressing presynaptic terminal (Fig. 4). Upon ligand activation, PIR-B gives rise to an inhibitory intracellular signal through its ITIM, thereby weakening the synaptic contact; ultimately leading to retraction of the terminal. At another more electrically active synapse, MHC class I expression is suppressed and the presynaptic terminal is therefore retained. In the case for axotomy-induced synapse plasticity, the following speculations can be made: when a motoneuron is axotomized, MHC class I molecules are induced, as previously shown (Maehlen et al., 1988; Maehlen et al., 1989; Linda et al., 1998; Linda et al., 1999). Positive gene inducers could in this case be IFN-γ released from activated glial cell or the lack of electrical activity. MHC class I molecules are translocated to the existing synapses on the surface of the motoneuron. Depending on the receptor repertoire on the different types of presynaptic terminals, the MHC class I molecules mediate either a stabilization signal at F-type terminals or a retraction signal at S-type terminals, thereby giving a selective synaptic elimination.

4.6 EXPRESSION OF COMPLEMENT PROTEINS IN THE SPINAL CORD

Complement proteins were recently implicated in developmental synaptic elimination of retinogeniculate projections (Stevens et al., 2007). In this publication, it was proposed that astrocytes induced a neuronal complement secretion, which targeted and

tagged presynaptic terminals, thereby labelling them for removal by adjacent glia. Interestingly, complement-deficient mice ($C1q^{-/-}$ and $C3^{-/-}$) showed a similar phenotype in the retinogeniculate system as the $TAP^{-/-}\beta_2m^{-/-}$ animals. Thus we explored the role for complement in adult synapse elimination seen in the spinal cord after a peripheral nerve lesion in **Paper III**.

Stevens et al. 2007 demonstrated mRNA expression of C1q in developing retinal ganglion cells. We found an up-regulation of C1q mRNA and IR as well as C3 IR in the spinal cord after axotmy. Due to difficulties in regular staining of complement proteins, Stevens et al. 2007 employed an unconventional form of immunostaining termed array-tomography. This experiment showed a punctate C1q and C3 staining pattern, sometimes associated with synaptic punctae. However, the subcellular localization of complement proteins remains unclear. Like Stevens et al, we found it hard to obtain a distinct staining pattern for C1q and C3 in the spinal cord using IHC. Both C1q and C3 were clearly up-regulated in axotomized ventral horn in proximity of the motoneurons, but high resolution double labelling images could not be obtained. The C1q expression appeared as a halo around the axotomized motoneurons and it was sometimes associated with synaptic terminals. The C3 pattern more resembled staining of activated glial cells and blood vessels.

4.7 A POTENTIAL ROLE FOR COMPLEMENT PROTEINS IN ADULT SYNAPSE ELIMINATION

In resemblance to the developing retinogeniculate system, adult $C3^{-/-}$ mice displayed a substantially increased retention of synaptic terminals on the somata of axotomized motoneurons compared to WT animals at 7 and 16 days after SNC (**Paper III**). This was shown by a reduced decrease in syph-IR in the DL motor pool in $C3^{-/-}$ compared to WT animals. This difference appeared to mainly consist of a retention of inhibitory terminals. The attenuated synapse elimination was then confirmed at the ultrastructural level where axotomized $C3^{-/-}$ motoneurons displayed a higher synaptic number and covering at 7 and 16 dpo. At 48 dpo, the WT group had recovered its synaptic covering and the groups were virtually the same. In parallel, the $C3^{-/-}$ animals displayed a stronger up-regulation of the growth associated protein 43 (GAP-43) mRNA, a regeneration associated protein, and a more rapid recovery of crude motor function after SNC. Taken together, this indicates that regeneration is more rapid in the absence of complement C3.

Since the effect on axonal regeneration seen in $C3^{-/-}$ animals is influenced by the immune system acting on the lesion nerve stump, we studied the influx of macrophages in the sciatic nerve. No apparent difference was seen between $C3^{-/-}$ and WT animals.

4.8 REACTIVE GLIOSIS IN MHC CLASS I AND COMPLEMENT C3-DEFICIENT MICE

Glial cells are activated after axotomy and are suggested to be involved in synaptic elimination (Blinzinger and Kreutzberg, 1968; Graeber et al., 1988; Tetzlaff et al., 1988; Svensson and Aldskogius, 1993; Aldskogius et al., 1999). It was therefore of particular interest to study glial reactivity in the ventral horn after nerve lesion. At one and two weeks after axotomy, there was a strong proliferation of microglia in WT animals, as visualized by IHC for Iba1 (microglial marker). No obvious difference was detected between WT and $K^{b-/-}D^{b-/-}$ or $C3^{-/-}$ animals. $TAP1^{-/-}\beta_2m^{-/-}$ animals displayed a moderate, but statistically significant reduction of Iba1 immunoreactivity (data not shown), which may indicate that non-classical MHC class I proteins are important in the regulation of microglial activation. This, however, needs to be confirmed with more quantitative methods. Astrocytic reactivity estimated by quantifying GFAP immunoreactivity did not reveal any difference between any of the groups or was inconclusive.

In a study by Stevens et al. 2007, astrocytes were implicated in a complement mediated developmental synaptic elimination process, while Shatz and co-workers propose a more strict neuron to neuron interaction. In the spinal cord the distinction between the role for glial cells contra neuron to neuron interactions remains unclear. Neither astrocytes nor glia display any morphological differences in the animals lacking MHC class I function or C3. It should however be emphasized that the methods used in this thesis may not reveal the true role of glial cells in synaptic elimination. Alternatively, this could also indicate a principal role for neuronal MHC class I and complement in this process, but more functional experiments and studies in conditional knock-outs will be required to further determine the role of immune molecules in different resident CNS cell types.

4.9 SYNAPSE ELIMINATION AND MOTONEURON RECOVERY AFTER LESION

The previous finding of a preferential retraction of glutamateric terminals from axotomized motoneurons led to the hypothesis that the severed neuron in this way could be protected from excitotoxicity (Linda et al., 2000). In this sense, the synapse elimination would serve a purpose and be protective. This notion is supported by the studies in MHC class I and complement-deficient mice where an accelerated loss and retention of synaptic terminals respectively, appear to correlate to the functional motor recovery. However, we have recently shown that the return of synaptic terminals during the recovery after SNC and SNR in WT mice does not appear to directly influence the crude motor recovery (Zelano et al., 2009). This would implicate that a fully restored synaptic input at the level of the cell body is not compulsory for a successful motor recovery. One explanation could of course be that a complete restoration of synaptic input to the cell body is imperative for a more refined motor control than the one tested in this study or that the remaining synaptic input can increase their activity in a compensatory manner.

In another perspective, axotomy-induced synapse elimination can be counteracted by providing neurotrophic support (Davis-Lopez de Carrizosa et al., 2009). Changes in synaptic covering of axotomized motoneurons may thus merely reflect the general state of the severed neurons.

4.10 CONCLUDING REMARKS AND FUTURE PERSPECTIVE

There is accumulating evidence that the nervous system and the immune system share several key molecules and signalling pathways. Recent findings of new roles for certain immune recognition molecules have opened a whole new exciting area of research. In the work presented in this thesis, we have focused on how two groups of immune recognition molecules are linked to plasticity and regeneration of spinal motoneurons.

Exaltation aside, there are certain concerns that need to be considered. As previously discussed, virtually all published *in vivo* studies use global null mutants for specific immune molecules where one cannot discern the specific effects in the nervous system from the effects in immune system. In this thesis, we have hypothesized that the central motoneuron reaction to axotomy is mainly dependent on nervous system expression since the BBB restricts the access for immune cells. In the studies of PNS plasticity, the immune system is obviously present. A possibility that has not been explored in this thesis is that the immune system may have other intimate interactions with the nervous system than during tissue inflammation or pathological conditions. Reports that normal adaptive immunity is required during CNS development and after spinal cord injury would lend support to this idea (Hammarberg et al., 2000b; Ziv et al., 2006).

Moreover, even if peripheral immune cells are more or less absent in the spinal cord during the conditions studied, it is still hard to dissect the specific roles for immune molecules in different glial cells and neurons. The difficulties in showing specific neuronal expression and subcellular localization for certain immune recognition molecules have made mechanistic *in vivo* studies hard to carry out, thereby leaving many logical gaps. One possibility would be *in vitro* studies; where one can isolate different cell types, but when studying complex interactions with multiple cell types or possibly even multiple systems *in vitro* experiments risk to fall short.

Conceptually, these issues may result in skepticism towards this new concept for nervous system interactions and must be dealt with before the broad scientific community will adopt it. The main conclusion is therefore, as previously discussed, that *in vivo* studies with more genetically tailored animals should be the next step. In addition, it would also be desirable with more cross-disciplinary collaborations since this field lies on the border between neuroscience and immunology; and a subspecialized scientist often tends to have too narrow of a mind. In that manner, this thesis has certainly made progress.

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