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IDENTIFICATION OF PATHWAYS OF DEGENERATION AND PROTECTION IN MOTOR NEURON DISEASES

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The image on the cover is a picture of a collection cap containing motor neurons that were isolated during one of my several LCM sessions.

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IDENTIFICATION OF PATHWAYS OF DEGENERATION AND PROTECTION IN MOTOR NEURON DISEASES

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By

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To my grandparents.

ABSTRACT

Motor neuron diseases preferentially affect specific neuronal populations with distinct clinical features even if disease-causing genes are expressed in many cell types. In spinal muscular atrophy (SMA), somatic motor neurons are selectively vulnerable to a deficiency in the broadly expressed survival of motor neuron 1 (*SMN1)* gene. In amyotrophic lateral sclerosis (ALS), mutations in multiple ubiquitously expressed genes have been identified that result in the same selective vulnerability. However, certain somatic motor neuron groups, including oculomotor and trochlear (CN3/4) neurons, are for unknown reasons relatively resistant to degeneration. We hypothesized that we could use CN3/4 motor neuron resistance as a tool to dissect mechanisms of vulnerability and protection, which would aid in identifying drug targets for the treatment of so far incurable motor neuron diseases.

Within this thesis work, we developed a robust method for spatial transcriptomic profiling of closely related neuronal populations that is sensitive down to single cells and can be applied to partly degraded human post-mortem tissues. We called this method LCM-seq (laser capture microscopy coupled with RNA sequencing). We applied LCM-seq to reveal longitudinal changes in gene expression in a mouse model of SMA in order to elucidate distinct adaptation mechanisms of several motor neuron populations that could account for their differential susceptibility. We revealed a common activation of DNA damage response and apoptosis pathways in somatic motor neurons independent of their susceptibility. We furthermore found gene expression changes that were preferential to the resistant CN3/4 motor neurons. Of particular interest were genes that function in regeneration, synaptic vesicle release and those that protect cells from oxidative stress and apoptosis. We speculate that these genes could play a role in the resistance of CN3/4 motor neurons and their manipulation in vulnerable motor neurons could be used to protect these from degeneration. As proof of concept, we further investigated candidates with implications for differential vulnerability that we had previously identified in a transcriptome analysis in the normal rat. We demonstrated a relative conservation across species by confirming the expression patterns of multiple proteins in mouse and human and in health and disease. Moreover, we provided functional evidence that the oculomotor restricted insulin-like growth factor 2 (IGF-2) can rescue vulnerable spinal motor neurons in *in vitro* and *in vivo* models of ALS. This indicates that IGF-2 could in part play a role in the preservation of CN3/4 motor neurons in ALS. By combining comprehensive studies in mouse models and the use of human ALS patient tissues as well as patient-derived induced pluripotent stem cell based *in vitro* assays we could maximize the chance of identifying mechanisms with relevance in human disease.

In conclusion, we provide a tool box for transcriptional profiling of neuronal populations with differential vulnerability followed by functional studies in mouse and human aiding in elucidating pathological mechanisms in neurodegenerative diseases, which could lead to the identification of drug targets for the treatment of motor neuron diseases.

LIST OF SCIENTIFIC PAPERS

I. Comley L*, Allodi I*, **Nichterwitz S***, Nizzardo M, Simone C, Corti S & Hedlund E (2015) Motor neurons with differential vulnerability to degeneration show distinct protein signatures in health and ALS. *Neuroscience* 291:216–229

II. Allodi I*, Comley L*, **Nichterwitz S***, Nizzardo M, Simone C, Corti S & Hedlund E (2016) Differential neuronal vulnerability identifies IGF-2 as a protective factor in ALS. *Scientific Reports* 6:25960, doi:10.1038/srep25960

III. **Nichterwitz S*,** Chen G*, Aguila Benitez J, Yilmaz M, Storvall H, Cao M, Sandberg R, Deng Q & Hedlund E (2016) Laser capture microscopy coupled with Smart-seq2 for precise spatial transcriptomic profiling. *Nature Communications* 7:12139; doi:10.1038/ncomms12139

IV. **Nichterwitz S,** Storvall H, Nijssen J, Comley L, Allodi I, Van der Lee M, Deng Q, Sandberg R & Hedlund E. (*Manuscript)* Resistant and vulnerable motor neurons display distinct transcriptional regulation in spinal muscular atrophy.

** These authors contributed equally to this work*

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LIST OF ABBREVIATIONS

ALS	Amyotrophic lateral sclerosis
CN	Cranial nerve
CN3	Oculomotor nucleus
CN4	Trochlear nucleus
CN7	Facial nucleus
CN10	Dorsal motor nucleus of cranial nerve X
CN12	Hypoglossal nucleus
IGF-2	Insulin-like growth factor 2
iPSC	Induced pluripotent stem cell
LCM	Laser capture microscopy/microdissection
MND	Motor neuron disease
NMJ	Neuromuscular junction
SC	Spinal cord
SMA	Spinal muscular atrophy
SMN	Survival of motor neuron protein
SOD1	Superoxide dismutase 1

Throughout this thesis, common nomenclature from the field was used when referring to proteins and genes, for example IGF-2 and TDP-43. In all other cases, official gene symbols were used. Capitalization and italics were used according to HUGO and MGI guidelines:

SYMBOL	Human gene
Symbol	Mouse gene
SYMBOL	Human and mouse protein

1 INTRODUCTION

Motor neuron diseases (MNDs) are characterized by the selective loss of somatic motor neurons that innervate skeletal muscle and control voluntary movements. Accompanied by massive muscle wasting leading to paralysis, these diseases are severely debilitating and mostly lethal. Death usually occurs due to respiratory failure and so far, there are no cures for any MNDs.

1.1 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset MND with one to three new cases per year in a population of 100,000. Onset of ALS typically occurs between 46 and 63 years of age and patients die on average between three and five years after diagnosis. Motor neurons originating in the cerebral cortex, brainstem, and spinal cord are affected in ALS. More specifically, limb-, trunk-, and diaphragm- innervating spinal motor neurons, hypoglossal motor neurons innervating tongue and esophagus, facial and trigeminal motor neurons, which control mimics and mastication, respectively, degenerate.

The majority of ALS cases appear sporadic (90-95%, sALS), whereas 5-10% are clearly inherited (familial ALS, fALS). More than 50 sALS- and fALS- associated genes have been identified so far, all of them being broadly expressed throughout many tissues. Among them are genes that function in several aspects of RNA metabolism, protein homeostasis and quality control, and cytoskeletal organization. The most commonly mutated gene in ALS (found in 25% of the familial and 10% of sporadic cases) is the recently identified *C9ORF72* (DeJesus-Hernandez et al. 2011; Renton et al. 2011), the exact function of which is still unclear. Among the most prevalent genes implicated in RNA dysregulation are the TAR DNA-binding protein (*TARDBP*, TDP-43) (Gitcho et al. 2008; Sreedharan et al. 2008; Kabashi et al. 2008) and fused in sarcoma (FUS) (Kwiatkowski et al. 2009; Vance et al. 2009), causing around 5% of fALS and less than 1% of sporadic cases each. Another important 'ALS gene' is superoxide dismutase 1 (*SOD1*), which is responsible for around 20% of familial cases and 3-7% of sALS cases. Mutated *SOD1* was the first ALS-causing gene identified in the early nineties (Rosen et al. 1993) and much of the knowledge about disease mechanisms originates from studies in mutant *SOD1* transgenic mouse models of ALS, which will be discussed later.

1.1.1 Disease mechanisms and genetics in ALS

Independent of the etiology of the disease, deregulation of many cellular processes can be observed but what is cause and effect remains mostly unclear. Oxidative stress, mitochondrial dysfunction, excitotoxicity, ubiquitin and proteasome dysfunction, activation of inflammatory and apoptosis pathways, RNA metabolism deficits, DNA damage and cytoskeletal abnormalities including axonal transport defects (**Figure 1.1**) have been observed in fALS as well as sALS (reviewed in Ferraiuolo et al. 2011; Taylor et al. 2016). Currently, the only

approved drug for the treatment of ALS is Riluzole, which, among other not fully elucidated functions, inhibits presynaptic glutamate release and thus reduces excitotoxicity. Excitotoxicity is just one of many disease mechanisms in ALS and thus it is not surprising that Riluzole elicits only moderately beneficial effects, extending the lifespan of patients by a few months only (Miller et al. 2012).

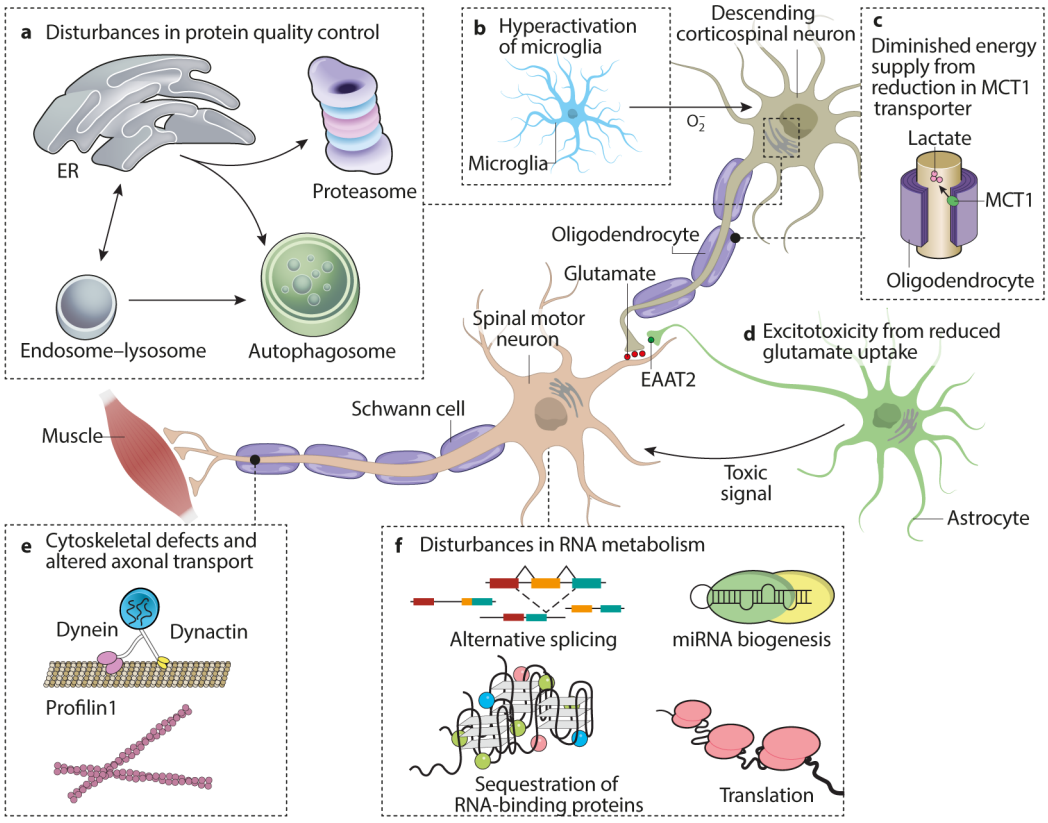


Figure 1.1. ***Many cellular processes are disturbed in ALS.*** (a) The protein quality control can be compromised in ALS. Mutations in autophagy genes (e.g. ***UBQLN2, OPTN*** and ***TBK1***) are responsible for familial as well as sporadic ALS cases and mutations in *SOD1* lead to misfolding and aggregation of the protein. Endoplasmatic reticulum (ER) stress is a common feature in ALS motor neurons. Glial cells are major contributors with (b) microglia releasing inflammatory cytokines, (c) a lack of metabolic support from oligodendrocytes and (d) astrocytes secreting toxic soluble factors as well as exacerbating excitotoxicity due to decreased uptake of excitatory neurotransmitters. (e) Cytoskeletal defects and altered axonal transport are a common and early feature of ALS. Furthermore, mutations in the gene encoding a tubulin subunit ***TUBA4A***, in **PFN1**, an actin binding protein, and in **DCTN1**, a component of the dynein motor complex, can cause ALS. (f) Multiple aspects of RNA metabolism are deregulated and mutations in several RNA binding proteins can cause ALS, such as **FUS, TDP-43, HNRNPA1, ANG** and **MATR3**. *Reprinted by permission from Macmillan Publishers Ltd: [NATURE] (Taylor et al. 2016), copyright (2016), license number 4053710699392. (Font size was adjusted to improve legibility).*

Protein aggregations are a hallmark feature of ALS that can be manifested in ubiquitinated cytoplasmic inclusions of several different proteins like SOD1, FUS and TDP-43. SOD1 is a cytoplasmic enzyme that converts harmful superoxide radicals to oxygen and hydrogen peroxide. Multiple mutations in the *SOD1* gene have thus far been identified to cause ALS, most of which confer conformational instability of the protein resulting in its aggregation but

also the formation of soluble oligomers. The molecular basis of how mutated SOD1 exerts its toxicity are not completely understood, however, a toxic-gain-of function is likely. Increased oxidative stress, mitochondrial dysfunction, defects in axonal transport, autophagy dysregulation and endoplasmatic reticulum (ER) stress have been linked to *SOD1* mutations. Both DNA/RNA- binding proteins FUS and TDP-43 play roles in transcription, splicing and micro RNA processing and mainly locate to the nucleus under normal physiological conditions. Both proteins target thousands of RNAs (Polymenidou et al. 2011; Tollervey et al. 2011; Lagier-Tourenne et al. 2012) and altered function of either FUS or TDP-43 may have broad consequences. A loss of its nuclear function is thought to be a major disease-causing mechanism for TDP-43 (Walker et al. 2015). However, a toxic gain-of-function cannot be excluded as contributor to disease pathology (Guerrero et al. 2016). Cytoplasmic TDP-43 containing inclusions can be found in the majority of both sporadic and familial ALS (except for SOD1 and FUS cases), suggesting a more general role in ALS pathology trough either loss- or toxic gain-of-function. As pathology occurs irrespective of normal FUS in the nucleus, mutations in this gene likely lead to a toxic gain-of-function (Mitchell et al. 2013; Sharma et al. 2016; Sun et al. 2015). At the same time, a loss-of-function mechanism also in FUS- ALS cannot be excluded as contributing to disease pathology: depletion of both FUS and TDP-43 results in transcription associated DNA damage *in vitro* (Hill et al. 2016). Activation of the DNA damage response can lead to apoptosis and it will be interesting to reveal if DNA damage is an early event in ALS pathology.

There is no consensus yet on the underlying mechanism also in *C9ORF72*-related ALS. ALS-causing *C9ORF72* presents with a vast hexanucleotide repeat expansion that results in the sequestration of RNA and proteins (RNA foci (DeJesus-Hernandez et al. 2011)) and the presence of dipeptide repeat proteins (DPRs) through repeat-associated non-ATG dependent translation (Mori et al. 2013). RNA foci might sequester RNA binding proteins and thus lead to dysregulation of several biological processes. Even though its function is not clear, there is emerging evidence for a role of C9ORF72 in nuclear import/export (Jovičić et al. 2015; Zhang et al. 2015; Freibaum et al. 2015), which is in line with the presence of TDP-43 positive cytoplasmic inclusions in C9ORF72 patients. More recently, DPRs have been linked to the mislocalization of TDP-43 (Khosravi et al. 2016). At the same time, decreased levels of normal C9ORF72 protein have been reported in patients (Waite et al. 2014) raising the possibility of additional loss-of-function mechanisms in C9ORF72-related ALS. However, the finding that neural depletion of *C9orf72* in mice does not produce a motor phenotype or altered survival suggest that loss-of-function alone is not a disease-causing mechanism (Koppers et al. 2015).

1.2 Spinal muscular atrophy

A different form of MND, with an incidence of 1/6000-1/10,000 live births and a carrier frequency of 1 in 50, is spinal muscular atrophy (SMA). SMA is the most common genetic cause of infant death and children with the most severe forms of SMA do not live past their

second year of life. Depending on the age of onset and the maximum motor function achieved by the patients, SMA is typically categorized into five types: type I-IV, with the most common and severe form being type I, and type IV being an adult-onset, non-lethal form. Extremely severe cases, where death occurs before 6 months of age, are classified as type 0 SMA. In SMA, predominantly motor neurons in the anterior horn of the spinal cord are affected. However, an increasing number of studies report the involvement of brainstem motor nuclei, such as the facial or hypoglossal nucleus (Harding et al. 2015; Petit et al. 2011; Rudnik-Schöneborn et al. 2009).

In contrast to ALS, which can be caused by mutations in one of many different genes, SMA is a monogenetic disease. In more than 95% of the cases, SMA is caused by homozygous deletions or mutations in the survival of motor neuron (*SMN*) 1 gene (Bussaglia et al. 1995; Lefebvre et al. 1995). The SMN protein is ubiquitously expressed and has functions in multiple aspects of RNA processing (reviewed in Singh et al. 2017). The complete loss of functional SMN protein is embryonically lethal (Schrank et al. 1997). Owing to a gene duplication that is almost exclusive to humans, this loss can be compensated for by the expression of the nearly identical paralogue gene *SMN2* (Monani et al. 2000). However, one base pair transition leads to exon 7 skipping in around 90% of the transcripts, thus resulting in a truncated form of the SMN protein, which is less stable (Monani et al. 1999). Humans possess a variable number of *SMN2* copies. A higher *SMN2* copy number can either prevent SMA completely or lead to a less severe form of the disease (Monani et al. 2000; Taylor et al. 1998). Increasing exon 7 inclusion in transcripts from the *SMN2* gene presents a most promising therapeutic strategy and encouragingly, positive outcomes in phase I and II clinical trials have already been reported (Chiriboga et al. 2016; Finkel et al. 2017).

1.2.1 SMA disease mechanisms: SMN- one gene, many functions

Several of the disease mechanisms that occur in ALS have also been described in SMA, such as mitochondrial abnormalities (Acsadi et al. 2009; Miller et al. 2016), oxidative and ER stress (Ng et al. 2015), neuroinflammation (Rindt et al. 2015), axonal transport defects and in particular RNA metabolism related disturbances. Due to the multifaceted function of SMN at several stages in RNA processing and additional functions, also here the exact mechanism(s) by which low levels of SMN result in SMA remains elusive.

The most studied function of the SMN protein is its role in the assembly of small nuclear ribonucleoproteins (snRNPs), essential components of the spliceosome. Several studies have correlated snRNP assembly activity with disease severity (Wan et al. 2005; Gabanella et al. 2007). A role for splicing defects in SMA is underlined by the finding that splicing alterations were identified in several models of SMA (Zhang et al. 2008; Bäumer et al. 2009; Zhang et al. 2013; Doktor et al. 2016). These alterations are not restricted to affected tissues, however, and it remains unclear to what extent dysregulation of splicing contributes to motor neuron pathology. Furthermore, there is no consensus about the timing of splicing

alterations (pre-symptomatic versus late-symptomatic changes) (Bäumer et al. 2009; Zhang et al. 2013) and thus neither on a causal or effective nature of splicing disturbances.

SMN may have tissue but also cell compartment specific functions and the detection of its presence in neuronal processes was quickly followed by studies investigating an axonal specific role (Jablonka et al. 2001; Fan and Simard 2002). Here, SMN functions in messenger ribonucleoprotein (mRNP) assembly for the axonal transport of transcripts including β-actin (*ACTB*) and *GAP43* mRNA (Rossoll et al. 2003; Fallini et al. 2010, 2016). Given that the neuromuscular junction (NMJ) is an early target in the pathology of SMA as evidenced by neurofilament accumulation at the presynaptic terminal (Cifuentes-Diaz et al. 2002), the function of SMN in axonal trafficking is a likely early contributor to disease. Furthermore, the lack of SMN-binding to the actin associated protein profilin 2 (PFN2) contributes to impaired neurite outgrowth (Bowerman et al. 2007) and implies yet another function for the SMN protein in cytoskeletal dynamics.

Apart from snRNP and mRNP assembly, SMN has been proposed to serve roles in many aspects of RNA processing, including transcription, translation and histone mRNA modification (Singh et al. 2017). A direct interaction of SMA with RNA polymerase II has already been described in 2001 (Pellizzoni et al. 2001) and recently a role for SMN in the R-loop resolution pathway for transcription termination has been suggested (Zhao et al. 2015).

Due to the diverse functions of the SMA-causing gene it still remains challenging to pinpoint the disease-causing mechanism(s). Most likely it is a combination of the lack of different functions that contributes to the overall pathology seen in SMA. While motor neurons appear to be the primarily affected cell type, disruption in other tissues is clearly evident (reviewed in Hamilton and Gillingwater 2013). Different tissues likely not only depend on different levels of SMN (Kariya et al. 2014) but also on different functions. To understand the susceptibility specifically of motor neurons, comparisons to other motor neuron diseases are a promising strategy.

1.3 Where do ALS and SMA meet?

The shared motor neuron pathology and continuously increasing overlap in disease mechanisms revealed between ALS and SMA make cross disease comparisons an attractive approach to understand selective vulnerability in MNDs. Interestingly, FUS can directly interact with SMN and mutations in FUS (that cause ALS) result in dysregulation of SMN as shown by nuclear gem depletion (of which SMN is a major component (Liu and Dreyfuss 1996)) (Sun et al. 2015) as well as altered axonal localization of the protein (Groen et al. 2013). Overexpression of *SMN* in TDP-43 ALS mice had beneficial effects on motor neuron preservation (Perera et al. 2016). Furthermore, yet another DNA/RNA binding protein has recently been associated with motor neuron disease. Two groups independently demonstrated that genetic depletion of the zinc finger protein *Zfp106* (*Zfp106*$^{-/-}$) in mice results in a

profound neuromuscular phenotype with motor neuron degeneration (Joyce et al. 2016; Anderson et al. 2016).

Taken together, the fact that several DNA/RNA binding proteins are associated with motor neuron diseases and DNA damage and alterations in RNA metabolism can have widespread consequences makes these mechanisms interesting targets for further investigation in the context of motor neuron susceptibility.

1.4 Differential vulnerability in motor neuron disease

As mentioned above, all of the known MND-causing genes are widely expressed in most tissues and cell types throughout the body but somatic motor neurons appear particularly vulnerable to these toxic genetic changes. Interestingly, there are striking differences in susceptibility between subtypes of somatic motor neurons. The reasons for this selective and differential vulnerability, however, are poorly understood. Among the relatively resistant populations, in both ALS and SMA, are the motor neurons of the oculomotor (CN3), trochlear (CN4) and abducens (CN6) nuclei (Gizzi et al. 1992), which control eye movements within the ocular motor system. Sparing of these cells enables the use of eye tracking devices as means of communication and navigation for patients that have otherwise lost the ability to move and speak (Kubota et al. 2000; Caligari et al. 2013).

1.4.1 Preservation of the ocular motor system

In both ALS and SMA, changes at the interface of neuron and muscle, the neuromuscular junction (NMJ), are early features of disease pathology. Defects in axonal transport occur in ALS before an overt phenotype can be seen (Bilsland et al. 2010), whereas NF accumulation and impaired neurotransmitter release can be observed at the NMJs of pre-symptomatic SMA mice (Ruiz and Tabares 2014; Kariya et al. 2008; Cifuentes-Diaz et al. 2002). In ALS, retraction of axons from muscle endplates occurs before a pronounced loss of motor neuron somas (‘dying back’ pattern (Fischer et al. 2004)). Muscles innervated by vulnerable motor neurons, including diaphragm and limb muscles show profound pathology already at early symptomatic stages, while NMJs in the extraocular muscles of SOD^{G93A} mice are preserved even at disease end-stage (Tjust et al. 2012; Valdez et al. 2012). This is consistent with findings in human post-mortem tissues, where extraocular muscles were substantially preserved compared to limb muscles of the same end-stage patients (Ahmadi et al. 2010). Comley et al. confirmed the persisting integrity of extraocular NMJs also in an SMA mouse model (Comley et al. 2016). In line with these findings, motor neuron counts were decreased in a number of vulnerable brainstem nuclei, in contrast to oculomotor neurons that were unchanged at disease end-stage in SOD1 and TDP-43 mouse models of ALS (Haenggeli and Kato 2002; Ferrucci et al. 2010; Spiller et al. 2016). The ocular motor system has a number of unique characteristics and several groups are trying to pinpoint which of these could explain its resistance. The transcriptome of CN3/4 motor neurons for example differs significantly

from other motor neuron populations as shown in rat, mouse, and man (Hedlund et al. 2010; Kaplan et al. 2014; Brockington et al. 2013).

1.4.2 Physiological properties of differentially vulnerable motor neurons

Differences in electrophysiological properties, like firing rate and input resistance of neuronal populations have been proposed to account for differential susceptibility among motor neurons. In ALS, fast firing (FF) motor neurons degenerate before fatigue resistant (FR) and slow (S) motor neurons (Frey et al. 2000; Hadzipasic et al. 2014; Pun et al. 2006). Relatively resistant S motor neurons have smaller dendritic trees (Cullheim et al. 1987) and thus a smaller membrane surface compared to FF and FR motor neurons. Hence, the input resistance of S motor neurons is lower resulting in higher excitability. In line with that, resistant CN3 neurons are more excitable than spinal motor neurons due to a higher resting membrane potential (Braughler and Hall 1982; Torres-Torrelo et al. 2012). In SMA, however, the comparison of NMJ pathology of muscles innervated by FF, FR, and S motor neurons revealed no correlation of motor neuron type and susceptibility to denervation (Murray et al. 2010, 2008). Thus, it is rather unlikely that the electrophysiological properties of CN3/4 motor neurons are solely underlying their resistance in all MNDs.

The same studies revealed differences in the survival of motor neurons that innervate muscles, which are in close proximity to each other. Consequently, also axon length can be ruled out as a key factor for susceptibility in MNDs.

To date, it is unclear why some motor neurons are more resistant to degeneration than others. Understanding the underlying basis for resistance and vulnerability could provide the basis for identifying drug targets for the treatment of MNDs.

1.5 Cell autonomous and non-cell autonomous mechanisms in motor neuron disease

As MND-causing genes are broadly expressed it appears fundamental to ask to what extent other cell types than the primarily affected ones play a role in disease.

1.5.1 Glia in motor neuron disease

Astrogliosis and microglial activation are hallmark features of ALS with reactive astrocytes and microglia surrounding degenerating motor neurons in ALS patients as well as mouse models (Hall et al. 1998; Perera et al. 2016; Henkel et al. 2004; Schiffer et al. 1996). Increased astrocyte activation has also been observed in SMA patients and mice (Rindt et al. 2015), and iPSC-derived SMA astrocytes display morphological abnormalities and altered calcium homeostasis (Rindt et al. 2015; Zhou et al. 2016). Mutant SOD1 astrocytes release inflammatory factors like prostaglandin E2, leukotriene B4 and nitric oxide (Hensley et al. 2006). Their important role in clearing excess excitatory neurotransmitters is compromised by the down-regulation of excitatory amino acid transporter 2 (EAAT2) thus contributing to excitotoxicity (Rothstein et al. 1995) (**Figure 1.1d**). Microglia appear to have an initially

protective function, whereas disease is later exacerbated by the release of cytokines from these cells (Zhao et al. 2013) (**Figure 1.1b**). Interestingly, microglia and astrocytes display markedly lower levels of activation around the resistant CN3 motor neurons at symptomatic and end-stages in SOD1 mice (An et al. 2014, and personal observation). Finally, important metabolic support for motor neurons from oligodendrocytes is lacking due to oligodendrocyte degeneration (Kang et al. 2013) and the downregulation of MCT1 lactate transporters (**Figure 1.1c**).

Studies using the selective manipulation of disease-causing genes in distinct cell types in ALS and SMA mouse models have gained us important insight on the role of glia in disease (Boillée et al. 2006; Kang et al. 2013; Yamanaka et al. 2008; McGovern et al. 2015; Kariya et al. 2008; Martinez et al. 2012). These reports suggest a strong impact of glial cells like astrocytes and microglia on disease progression. Initiation of degeneration, however, seems to come from within motor neurons and in ALS also from oligodendrocytes. Removal of mutant *SOD1* from motor neurons resulted in significantly delayed onset of the disease and early disease progression, whereas its selective depletion in microglia or astrocytes slowed disease progression substantially. While oligodendrocytes appear to be unaffected by low levels of SMN (O'Meara et al. 2017), restoration of normal SMN levels in Schwann cells can rescue peripheral myelination defects and improve NMJ pathology (Hunter et al. 2013). Survival, however, remains unaltered in these mice.

In general, simultaneous manipulation of the disease-causing gene in motor neurons and glia leads to a more pronounced effect on phenotype than targeting motor neurons alone. Thus, therapeutic intervention targeting glia presents a promising strategy to slow disease progression. This is exemplified by Mastinib and Ibudilast both of which reduce microglia secreted inflammatory factors. Mastinib was shown to slow disease progression in a SOD1 rat model of ALS even when administered after onset of paralysis through ameliorating neuroinflammation (Trias et al. 2016). Ibudilast has been used for more than two decades for the treatment of asthma. Its neuroprotective effect *in vitro* from cell death induced by microglia (Mizuno et al. 2004) gives hope for the treatment of neurodegenerative diseases. Both of these compounds are currently in phase II clinical trials in ALS (https://clinicaltrials.gov/).

1.5.2 Muscle in motor neuron disease

The relationship of muscle and motor neurons is characterized by communication into both directions. The extent to which the postsynaptic muscle is a driver of disease in ALS is highly debated. Also here, selective expression or depletion of mutant genes in muscle was used to assess the extent of the resulting motor phenotype. Miller et al. (2006) found functional improvement in mice where mutant *SOD1* was reduced in both motor neurons and muscle, whereas muscle-restricted reduction of mutant SOD1 protein (by 25% to 50% using two different strategies) had no beneficial effects. These findings indicate that either muscle is not

a driver of motor neuron degeneration in ALS, or that the reduction of mutant protein achieved was not sufficient to rescue the phenotype. Another study selectively overexpressed mutant *SOD1* in muscle, which resulted in muscle atrophy while motor neuron numbers were unaltered (Dobrowolny et al. 2008), rendering it unlikely that muscle is a systemic driver of disease. In contrast, the study by Wong and Martin (2010) demonstrated a severe motor phenotype including motor neuron degeneration when overexpressing mutant or wild-type SOD1 in muscle. These opposing findings can likely be attributed to the different strategies that were used to drive muscle-specific expression in these studies (lentiviral delivery directly to muscle by Miller versus use of the α-actin promoter by Wong and Martin) and further investigation is necessary to draw stronger conclusions as to what extent muscle is involved in the pathology of ALS.

To study the role of muscle in SMA, Cifuentes-Diaz et al. (2001) performed muscle selective *Smn*- depletion using the human skeletal actin (HSA) promoter and reported reduced survival of these animals. As mentioned above, complete depletion of SMN is embryonically lethal. These mice did not harbor any human *SMN2* transgene and the complete absence of SMN protein in muscle could therefore account for the disease phenotype. In support, restoration of SMN levels selectively in muscle on an SMA background (using the same promoter) did not result in increased life-span of the animals and neither was motor function improved. However, expression driven by the HSA promoter does not affect SMN expression in satellite cells. Satellite cells are skeletal muscle precursor cells that are responsible for muscle growth during early postnatal development. They are maintained in adulthood and are the basis for the massive regenerative capacity of muscle. Selective restoration of SMN in muscle progenitors with the Myf5 promoter led to normal muscle fiber growth and moderately extended survival (Martinez et al. 2012). However, no phenotypic improvement at the NMJ could be detected. In contrast to muscle specific restoration, selective normalization of SMN levels in motor neurons in the same study rescued transmission at the NMJ with only partial improvement of muscle fiber growth and a comparable increase in survival. Two studies that were co-published by the same lab (Iyer et al. 2015; McGovern et al. 2015) came to similar results demonstrating that muscle specific restoration or depletion did not affect survival, while normal SMN levels in motor neurons, even though survival effects were minimal, rescued electrophysiological deficits in SMA mice. The best results were achieved when restoring SMN levels through a nestin promoter, which drives expression in various precursor cells in the central nervous system, underlining the contribution of glia and other neuronal populations in the pathology of SMA.

1.5.3 Motor neuron intrinsic vulnerability

Selective introduction of disease-causing mutations in motor neurons on a normal background or selective restoration on a mutant background do have profound effects on motor neuron survival and/or motor function. Moreover, several *in vitro* studies show clear evidence that mutant motor neurons are less viable and more susceptible to mutant astrocyte-

mediated toxicity compared to wild-type motor neurons (Corti et al. 2012; Sareen et al. 2012; Di Giorgio et al. 2007; Ebert et al. 2009; Kiskinis et al. 2014; Nagai et al. 2007). It is therefore crucial to elucidate motor neuron intrinsic mechanisms in order to understand their selective vulnerability in MNDs.

1.6 Models of motor neuron disease

From electrophysiological measurements in motor neuron disease patients, analysis of human post-mortem tissues, and whole genome sequencing we have gained much insight into the underlying pathology and genetics in MNDs. Most of the knowledge on cellular and molecular disease mechanisms originate from studies that use animal or *in vitro* models of MNDs. ALS and SMA are successfully studied in models as diverse as zebrafish, *drosophila*, *C. elegans*, and yeast (reviewed in Edens et al. 2015; Patten et al. 2014; Tenreiro et al. 2013; Matsumoto et al. 2004). As the model systems used in this thesis are mouse models and stem cell-derived motor neurons, these will be discussed in more detail here.

1.6.1 ALS mouse models

Mutations in the *SOD1* gene were the first identified genetic cause of ALS (Rosen et al. 1993), which led to the fast development of rodent ALS models. *SOD1* knockout or expression of transgenes harboring mutations that reduce SOD1 activity in mice fail to induce a motor phenotype. In contrast, transgenic mice overexpressing different forms of mutated human *SOD1* develop a progressive motor neuron disease that closely resembles the pathology in human ALS. Mouse models with different mutations in the *SOD1* gene vary in disease progression rate, not in the general phenotype however. A widely used SOD1 model for ALS harbors a G to A transition in position 93 (SOD^{G93A}). SOD^{G93A} mice develop hindlimb paralysis and die at 4-5 months of age (Gurney et al. 1994). The severe neuromuscular phenotype of these mice is accompanied by astrocyte and microglial activation, mitochondrial abnormalities, altered autophagy pathways and ER stress recapitulating many features of the human disease. Importantly, studies investigating different motor neuron populations, that are known to be affected in human ALS patients, revealed a significant loss of relevant populations also in different SOD1 models (Haenggeli and Kato 2002; Kashlan et al. 2015; Ferrucci et al. 2010; Nimchinsky et al. 1999). At the same time, the relatively resistant CN3/4 motor neurons are spared. In general, mutant SOD1 mice are considered models for fALS. However, from the analysis of patient material it appears that the pathology and pattern of selective motor neuron vulnerability is similar in fALS and sALS (Shaw 1997; and reviewed in Peters et al. 2015), thus justifying the use of the available fALS models to understand sALS. We thus chose to use the SOD^{G93A} mouse model in studies I and II.

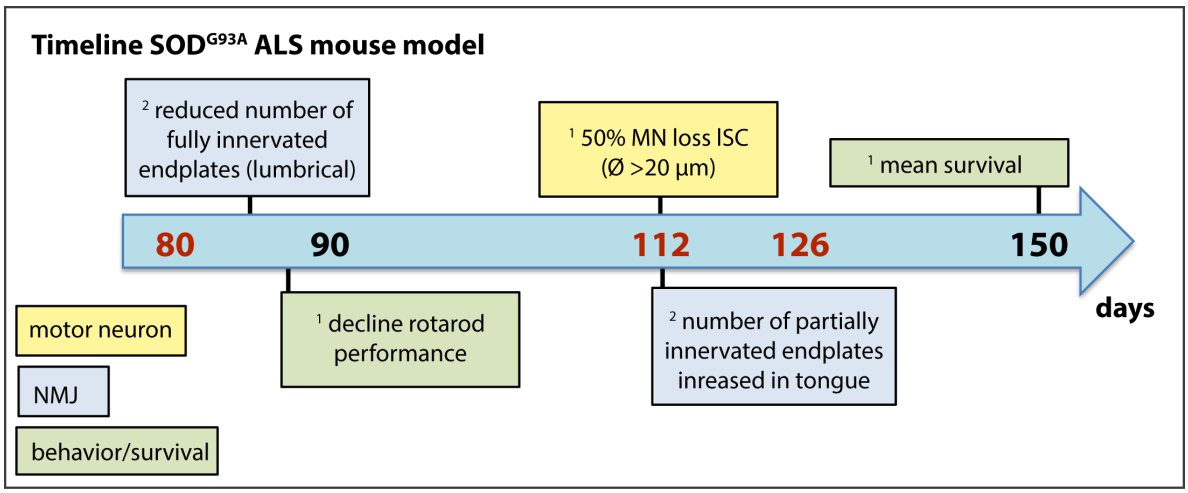


Figure 1.2. ***Timeline of SOD^{G93A} ALS mouse model.*** Approximate times for SOD^{G93A} overexpressing mice on a C57Bl/6J background. Lumbrical muscles of the hindlimbs show NMJ denervation at 84 days of age. Between 84 and 91 days, a decline in rotarod performance compared to littermate controls is evident. At the age of 112 days, around 50% of lumbar spinal (lSC) motor neurons with a diameter larger than 20 µm are lost and the number of only partially innervated endplates in the tongue is increased. Mean survival for females is 153±1.7 days and males 147±1.6 days. Red numbers indicate timepoints investigated in studies I and II. Mice in study II were treated at the age of 80 days, immunohistochemistry was performed on symptomatic animals at 112 and 126 days. NMJ = neuromuscular junction; MN = motor neuron. ([1]Mancuso et al. 2012; [2]Comley et al. 2016)

1.6.2 SMA mouse models

SMA is a monogenetic disease caused by the homozygous loss of functional *SMN1*. Mice only possess one gene encoding SMN (*Smn*) and its homozygous depletion is embryonically lethal (Schrank et al. 1997), whereas heterozygous depletion results in only a mild phenotypic change in the mice without reported effect on survival (Balabanian et al. 2007; Jablonka et al. 2000). The embryonic lethality of complete SMN deletion can be rescued with the homozygous introduction of the human *SMN2* gene ($Smn^{-/-}/SMN2^{+/+}$ (Monani et al. 2000)). These mice die at around postnatal day 6 and are considered a model of severe SMA (type I). Introduction of eight copies of *SMN2* results in a complete rescue of the phenotype, which is in line with the disease-modifying role of *SMN2* in human SMA. Another widely used mouse model of SMA has an additional transgene coding for the truncated version of the human *SMN* gene ($Smn^{-/-}/SMN2^{+/+}/SMN\Delta7^{+/+}$ (Le et al. 2005)) resulting in a slight extension of survival to 13-14 days. We chose this mouse model for study IV as it is still considered a model for severe SMA but allows more time for longitudinal investigation of disease mechanisms. Moreover, we have previously demonstrated the preservation of NMJs of CN3/4 motor neurons in this mouse model (Comley et al. 2016).

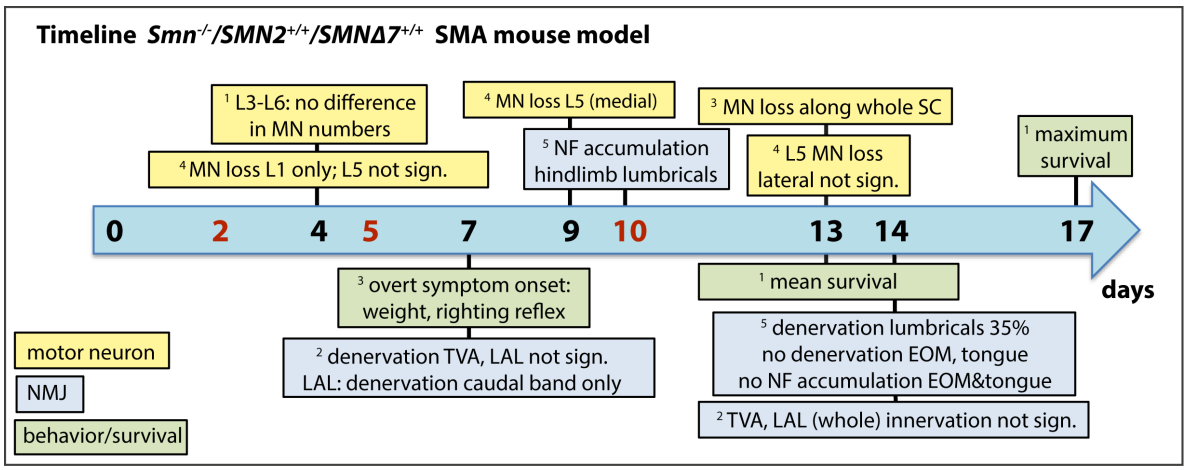


Figure 1.3. ***Timeline of 'delta7' mouse model of SMA.*** Motor neurons that innervate proximal and axial muscles (L1 and medial motor columns) are lost before motor neurons of the lateral motor columns that innervate limb muscles. Within the LAL, caudal and rostral bands show differential vulnerability. SMA mice display overt motor/behavioral symptoms from around seven days onwards: their righting reflex is impaired and SMA mice are significantly smaller than their healthy littermates. L = lumbar levels of the spinal cord; MN = motor neuron; sign. = statistically significant; TVA = transversus abdominus muscle; LAL = levator auris longis muscle; NF = neurofilament; EOM = extraocular muscle; NMJ = neuromuscular junction. Numbers in red indicate the time points investigated in study IV. ([1]Le et al. 2005; [2]Murray et al. 2008; [3]Bäumer et al. 2009; [4]Mentis et al. 2011; [5]Comley et al. 2016)

1.6.3 Cell culture models of ALS and SMA

In vitro models of MNDs have been used extensively to study disease mechanisms and to test the efficiency of potential drugs. Primary motor neurons and other disease relevant cell types can be obtained from wild-type animals or transgenic animal models. The development of protocols for the derivation of relevant cell types from mouse and human embryonic stem cells has facilitated the use of *in vitro* models. Vulnerable spinal motor neurons have been successfully generated from stem cells by recapitulating events occurring during embryonic development (reviewed in Allodi and Hedlund 2014). Pluripotent stem cells are initially expanded and subsequently embryoid body (EB) formation is induced. EBs can form all three germ layers, neuroectoderm, mesoderm and endoderm. Spinal motor neurons are generated from progenitors through extrinsic signals that are present in a distinct gradient along the rostro- caudal and dorso- ventral body axes. More specifically, cervical spinal motor neurons are patterned by Sonic hedgehog (Shh), which is required for ventralization, and retinoic acid (RA), which mediates caudalization. Addition of Shh and RA to the cell culture medium induces the generation of several neuronal populations, including Hb9- and Islet1/2- positive motor neurons. With the development of induced pluripotent stem cells (iPSCs) from patient fibroblasts these *in vitro* models have gained even more relevance.

iPSC-derived patient motor neurons and primary motor neurons from SMA mice have shorter neurites and/or axons (Rossoll et al. 2003; Corti et al. 2012). Moreover, SMA patient-derived motor neurons present a cell autonomous degeneration after six to eight

weeks in culture (Corti et al. 2012; Sareen et al. 2012). These characteristics can be easily assessed and used for the evaluation of disease modifiers such as IGF-2 in study II.

Reactive astrocytes surround degenerating motor neurons in ALS patients as well as mouse models. When culturing motor neurons with mutant SOD1 astrocytes derived from transgenic mice, these astrocytes exert a toxic effect on motor neurons via soluble factors (Di Giorgio et al. 2007; Marchetto et al. 2008) (**Figure 1.4**). This is also true for astrocytes derived from familial and sporadic ALS patients (Meyer et al. 2014; Haidet-Phillips et al. 2011). Furthermore, astrocytes remove excess of the excitatory neurotransmitter glutamate via EAAT2 (GLT-1), which is down-regulated in ALS. Glutamate excitotoxicity has been implicated in ALS (Van Damme et al. 2007; Bruijn et al. 1997; Howland et al. 2002) and glutamate-overload of motor neurons in culture leads to increased cell death (Hedlund et al. 2010). Both glutamate-overload of motor neuron cultures as well as co-culturing motor neurons with mutant astrocytes have therefore become widely used tools to model ALS-like toxicity *in vitro*.

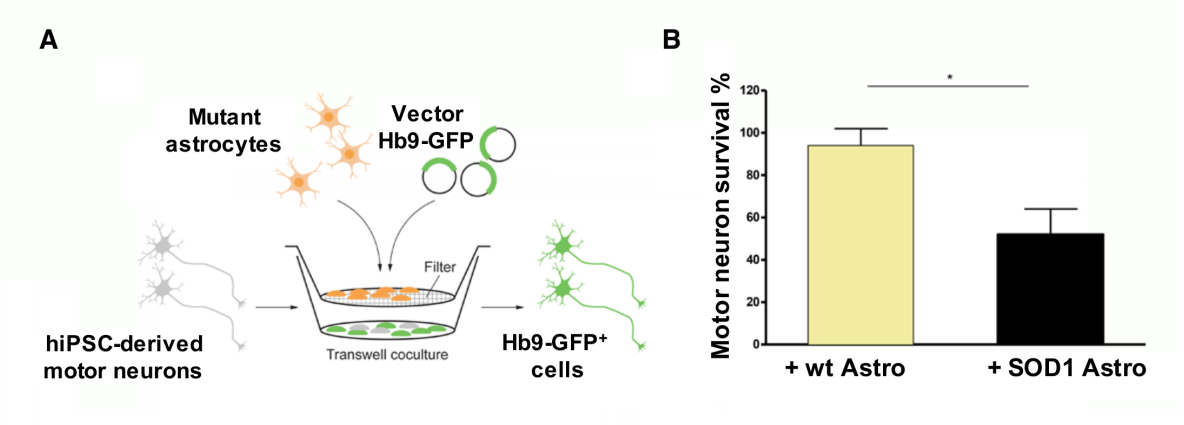


Figure 1.4. ***Mutant SOD1 astrocytes are toxic to motor neurons in vitro.*** (A) Schematic illustration of the co-culture system of astrocytes and human iPSC-derived motor neurons. Astrocytes are separated from motor neurons through a filter that allows for diffusion of soluble factors. Motor neurons are transfected with an Hb9-GFP construct to enable easy visualization of Hb9-positive cells. (B) Co-culture with mutant astrocytes significantly decreases motor neuron survival compared to co-culture with wild-type astrocytes. Student's *t*-test, two-tailed, * = $P<0.05$.

1.7 Disease mechanisms with a spotlight on motor neurons: a transcriptomics approach

It is necessary to define alterations in pathways upstream and downstream of the disease-causing gene to understand why some cell types are more vulnerable to degeneration than others. The use of transcriptomics presents an elegant way of dissecting such pathways by revealing gene expression changes in a tissue, cell population, or even a single cell. Several transcriptomics studies using different approaches have been conducted thus far and will be discussed here with emphasis on their experimental strategies.

1.7.1 Studies comparing disease versus health

Early studies in SMA mouse models used whole spinal cord tissue to extract total RNA and compare gene expression and splicing to wild-type littermate controls at different disease stages (Murray et al. 2010; Bäumer et al. 2009; Zhang et al. 2008). However, cell type specific adaptation mechanisms cannot be distinguished with this approach as gene expression and splicing changes could be masked or equaled out by the numerous cell populations present in the spinal cord. To circumvent this problem, transcriptional profiling of enriched motor neurons isolated by laser capture microdissection (LCM) can be used. Among the common themes detected in such studies investigating ALS motor neurons and their respective healthy controls, were cell communication, cellular growth, energy metabolism, the untranslated protein response (UPR), and ER stress (Lobsiger et al. 2007; Ferraiuolo et al. 2007; Saxena et al. 2009; Bandyopadhyay et al. 2013). The activation of ER stress pathways was also revealed in a transcriptomics study using iPSC-derived motor neurons from SMA patients (Ng et al. 2015). Importantly, Ng et al. could demonstrate the moderate but yet positive effect of ER stress inhibitors in a mouse model of SMA. A drawback of these studies is however, that it is impossible to distinguish general adaptation mechanisms from detrimental responses that lead to the degeneration of selectively vulnerable cells.

1.7.2 Studies comparing cell populations with differential vulnerability

A different approach from comparing disease versus health is to compare vulnerable versus resistant cell populations. An early study by Hedlund et al. (2010) investigated differences in the transcriptomes of resistant CN3/4 and vulnerable hypoglossal and spinal motor neurons from LCM dissected cells of wild-type rats. Similarly, Brockington et al. (2013) compared LCM dissected CN3 motor neurons with spinal motor neurons from human post-mortem tissues from neurologically normal controls. Both studies found substantial intrinsic differences in these cell populations that could account for their differential susceptibility. A similar approach was undertaken by Kaplan et al. (2014) where 'susceptibility and resistance candidates' were identified by comparing two resistant motor neuron pools with vulnerable spinal motor neurons. One of their susceptibility candidates, MMP-9, had been shown earlier to be present in spinal motor neurons of ALS patients and its absence extended the life-span of SOD^{G93A} mice (Lim et al. 1996; Kiaei et al. 2007; Kaplan et al. 2014). Yet another study used a backlabeling technique to identify cells in the spinal cord that innervate different muscles consisting of either fast- or slow-twitch fibers, which are differentially affected in ALS (Saxena et al. 2009). The labeled motor neurons were isolated using LCM and subjected to microarray analysis. Upon identifying ER stress as an important disease mechanism, the investigators revealed that treatment of these mice with the ER stress-protective agent salubrinal ameliorated disease pathology and extended average survival significantly. Importantly, Kiskinis et al. (2014) could confirm ER stress as a major disease mechanism in motor neurons derived from human patient iPSCs with *SOD1* mutations. Their analysis was

further extended to motor neurons derived from patients with sALS and C9ORF72 ALS patients. Among many differences between motor neuron transcriptomes, an overlap of gene expression changes associated with intracellular transport, oxidative stress and the UPR was found, indicating common mechanisms independent of the etiology of the disease. Taken together, these studies highlight the value of using differential susceptibility as a strategy to identify promising target mechanisms. When Kaplan and Saxena studied gene expression in the context of differential vulnerability, they were limited to the use of microarrays, which restricts the detection of genes to a given set of probes. RNA sequencing, however, opens up the possibility to reveal previously unknown transcripts and thus presents a valuable improvement to such strategies.

In summary, transcriptional profiling of motor neurons can provide important insight into disease mechanisms. Including pre-symptomatic and symptomatic disease stages within a longitudinal analysis could aid in dissecting causative from consequential alterations in cellular biological processes. Furthermore, these findings demonstrate that identifying transcriptome alterations in vulnerable as well as resistant motor neuron populations can lead to *de novo* identification of detrimental and protective disease mechanisms and even potential drug targets.

Given the variety of mechanisms implicated in ALS and the numerous functions of the disease-causing gene in SMA, it appears likely that the most promising treatment strategy will be a combination of factors rather than targeting only one aspect of motor neuron degeneration. Within this thesis work, we sought to develop a tool box and establish a comprehensive catalogue that can aid in understanding motor neuron susceptibility in ALS and SMA. We are exploring both, pathways of vulnerability and resistance, by first identifying them in mouse models of MND with the help of transcriptional profiling of neuronal populations with differential vulnerability. Confirmation on the protein level is initially achieved by immunohistochemistry on mouse tissues and subsequently in ALS patient tissues to evaluate their relevance for the human disease. With the same goal in mind, we use human iPSC-derived motor neurons to test the effect on survival of selected candidates. Lastly, promising candidates identified in the *in vitro* system are being evaluated in *in vivo* mouse models of MND (**Figure 1.5**).

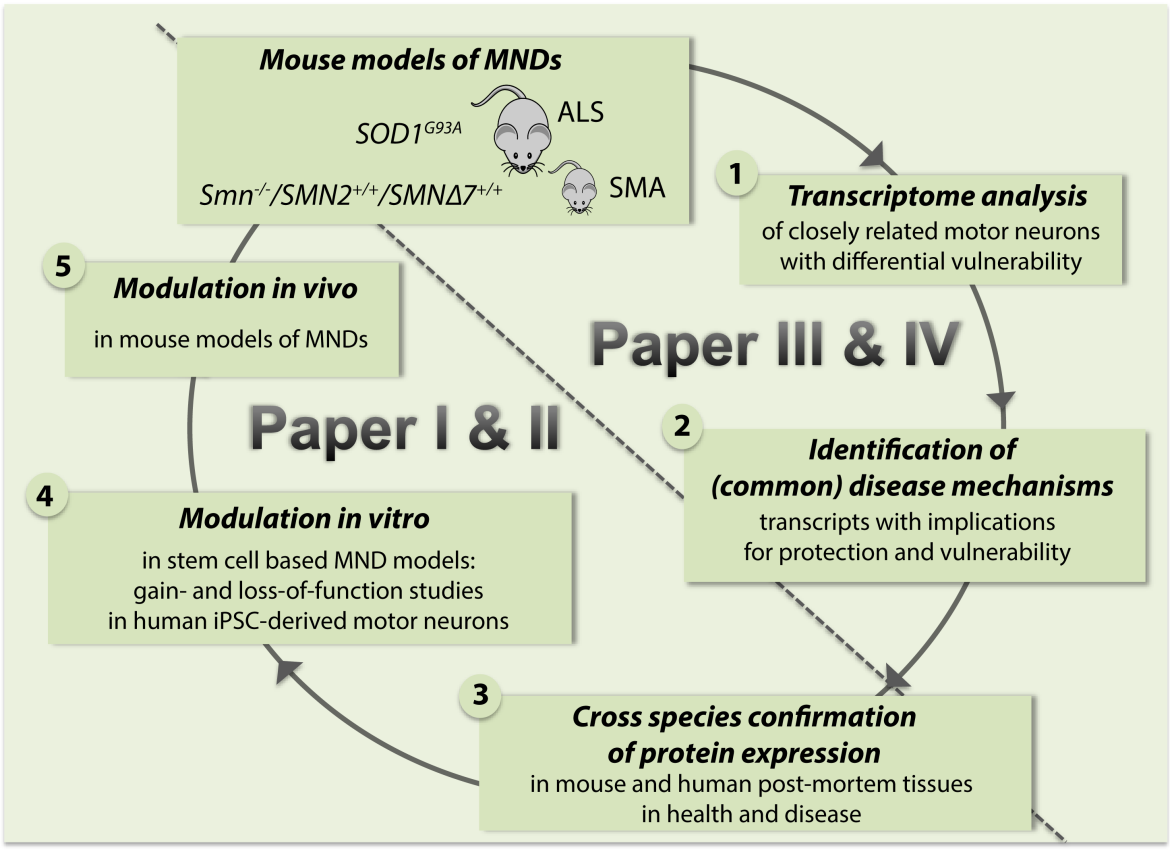


Figure 1.5. ***Identification of pathways of degeneration and protection in motor neuron diseases.*** Workflow overview.

2 AIMS

The overall aim of this thesis was to understand differential neuronal vulnerability in ALS and SMA and thereby elucidate underlying protective and detrimental adaptation mechanisms. We hypothesize that we can use oculomotor resistance as a tool to identify promising drug targets for the treatment of these so far incurable diseases.

Paper I. To identify a protein signature for differentially affected motor neurons in ALS and investigate the conservation across species.

Paper II. To demonstrate that oculomotor specific expression can be used to identify candidates that protect vulnerable motor neurons from degeneration in models of motor neuron disease.

Paper III. To provide a robust and reliable protocol for spatial transcriptomic profiling that can be applied to scarce neuronal populations and human post-mortem tissues.

Paper IV. To shed light on transcriptional adaptation mechanisms in differentially affected populations in SMA that could underlie their susceptibility to degeneration.

3 METHODOLOGICAL CONSIDERATIONS

3.1 Mouse and human motor neuron disease models

The translation of results achieved in mouse models of ALS and SMA to human clinical trials in general has been rather disappointing. While several mouse models recapitulate the human diseases fairly robustly, differences in phenotypes still prevail. SMA mouse models for example display severe defects in heart and liver tissues that are not typically observed in patients (apart from very severe cases, reviewed in Hamilton and Gillingwater 2013) that may alter disease progression significantly and appropriate read-outs have to be carefully considered. The previous limitation to mouse and rat models that are based on mutations in only one of many ALS genes (*SOD1*) has certainly contributed to the lack of clinical translation. The recently developed new mouse models based on several other ALS-causing genes such as *FUS* (Mitchell et al. 2013; Sharma et al. 2016)*, TARDBP* (TDP-43, Walker et al. 2015; Spiller et al. 2016) and *C9ORF72* (Liu et al. 2016) will benefit the translation of pre-clinical findings to human ALS. Another major problem is that due to its similarity to other neurological diseases at early stages, ALS is difficult to diagnose. Thus, patients recruited for clinical trials are typically at late stages of the disease when intervention might already be too late. Neurofilaments are the currently only reliable biomarkers for the diagnosis of ALS (Lu et al. 2012, 2015; Turner et al. 2013) and the further development of biomarkers will help overcome this problem.

The strength of the methods applied in this thesis work lies in the combination of comprehensive studies in mouse models and the use of human ALS patient tissues as well as *in vitro* assays with human iPSC-derived motor neurons. We can thus reduce the number of animals used and at the same time maximize the chance of identifying mechanisms with relevance for the human disease. We use an animal *in vivo* system to identify candidates that could confer vulnerability and resistance (study IV). To evaluate if these candidates are persistently expressed in disease and could thus play a role in motor neuron susceptibility, we use immunohistochemistry on mouse tissues from MND models as shown in studies I and II. Subsequently, the relevance for the human disease is evaluated by performing immunohistochemistry on human control and patient tissues. Candidates that pass the initial screen need to be functionally tested. We decided to test the effect of IGF-2 on motor neuron survival in study II in two human iPSC- based *in vitro* models of ALS. The use of patient iPSC-derived motor neurons does not only allow us to evaluate the relevance for the human disease, it furthermore allows us to study disease modifiers in sporadic ALS. Finally, promising candidates are tested in mouse models of MNDs, as shown in study II, in order to evaluate effects on disease progression and survival *in vivo*.

3.2 Advantages and limitations of laser capture microdissection coupled with RNA sequencing

RNA sequencing is a powerful tool to understand the transcriptome of a cell or cell population and thus learn about biological processes and disease mechanisms. Paper III in

detail describes the LCM-seq method that we subsequently used in study IV. Here, I would like to elaborate on the advantages and limitations of using LCM-seq in the SMA transcriptomics study.

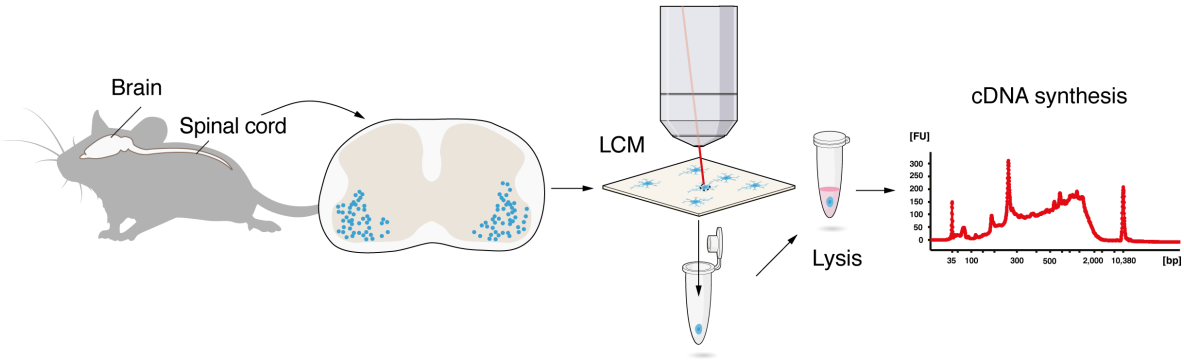


Figure 3.1. ***Overview LCM-seq workflow.*** Brain and spinal cord tissues are rapidly dissected and snap frozen. Coronal sections are prepared in a cryostat at -20°C (at a thickness which corresponds approximately to the diameter of the cells of interest) and mounted onto pre-cooled LCM membrane slides. Sections are stained with a quick histological staining immediately before LCM to visualize cell somas. Individual cells are cut with a laser and collected in dry PCR tubes. Cells are lysed in a mild hypotonic lysis buffer and cDNA libraries are prepared directly in the same tube using the Smart-seq2 protocol. Quality of cDNA libraries is evaluated with a Bioanalyzer (Agilent) before sequencing library preparation. Amount of cDNA in FU = fluorescent units; fragment size in bp = base pairs. LCM = laser capture microdissection.

3.2.1 Laser capture microdissection for the isolation of neuronal populations

For study IV, we wanted to compare the transcriptomes of six different neuronal populations, namely motor neurons of the spinal cord, motor neurons of the oculomotor and trochlear nuclei, the facial nucleus, the hypoglossal nucleus and the dorsal motor nucleus of the vagus nerve, as well as neurons of the red nucleus. There are no genetic markers that reliably distinguish all our populations of interest, making genetic labeling and the widely-used fluorescence-activated cell sorting (FACS) an unsuitable method for cell isolation in our study. Furthermore, brain and spinal cord tissues from postnatal mice do not allow for easy dissociation that is necessary for FACS, due to increased myelination and long cellular processes. Therefore, our method of choice was laser capture microdissection (LCM). For LCM, tissue was snap frozen immediately after dissection, followed by sectioning in the cryostat in order to prevent RNA degradation. Due to their large soma size and distinct location in the brainstem and spinal cord, we can easily identify motor neurons and neurons of the red nucleus using a histological staining. This staining is commercially available and optimized for RNA analysis after LCM (histogene, Arcturus). For laser dissection, each cell is being outlined individually, closely to the margins of the cell, in order to keep the inclusion of contaminating cells to a minimum (**Figure 3.2**).

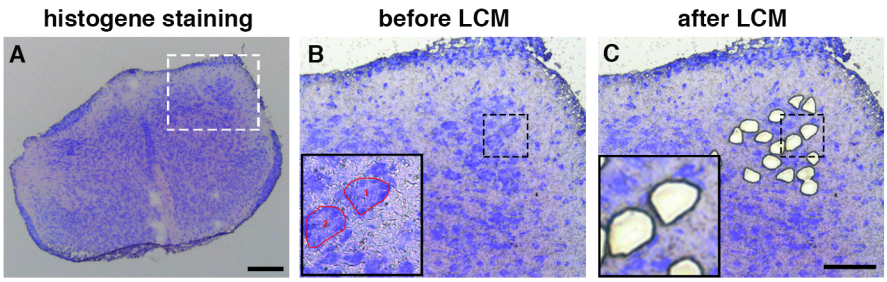


Figure 3.2. ***LCM for isolation of highly enriched neuronal populations.*** (A) Whole spinal cord section stained with histological staining (histogene) to visualize cell somas; rectangle indicates area that is shown in (B). Inset: cutting outlines (red) are drawn closely around the cell in order to minimize the inclusion of surrounding cells. Only cells with clear nucleus and nucleolus are selected in order to avoid capturing cells that might lie underneath the motor neuron. (C) Tissue after laser dissection. Scale bar in A = 200 µm, in C (applicable to B and C) = 100 µm.

One significant limitation of using LCM in our study is that we are restricted to investigating RNA species in the cell soma. Motor neurons possess long axonal processes and mRNAs are highly abundant all the way to the presynaptic terminals of these cells. Given the role of SMN in axonal RNA regulation, it would be interesting to include these RNAs in our analysis to gain insight into potential SMA-induced alterations in their regulation. Transcriptional profiling of LCM dissected NMJs has been performed previously (Ketterer et al. 2010), it remains challenging however, to identify motor neuron-derived transcripts in this setup as a substantial proportion of the dissected material derives from muscle tissue. *In vitro* microfluidic systems present an elegant way to circumvent this problem (Southam et al. 2013; Saal et al. 2014; Briese et al. 2015). Here, muscle cells and motor neurons can be co-cultured in separate compartments that are connected by micro-channels and thus only allow axons to cross. Due to the small amount of material isolated from crossing axons in the micro-channels, technical challenges remain also here.

3.2.2 Smart-seq2 for sequencing library preparation from laser capture microdissected cells

For optimal RNA sequencing results, large amounts of high quality input material are required. Recently, sequencing of single cells has become a widely-used method to explore the heterogeneity of cell types and identify new cell types and thus protocols for extremely low input material have been developed. We chose to use a single cell protocol for RNA sequencing library preparation to keep the number of cells collected comparably low. Previous studies applying LCM coupled with RNA sequencing use 200 – 4000 cells and sometimes required pooling of cells from several animals (Kadkhodaei et al. 2013; Zhang et al. 2013; Bandyopadhyay et al. 2013; Murray et al. 2015). Low input material, however, comes at the cost of increased technical variation and bias due to the necessity of amplification of the material. In particular, low abundant transcripts might be lost during exponential PCR amplification cycles. Nevertheless, using a protocol for low input material means less time spent for collection per sample and thus more samples collected in the end.

In this way, we reduced technical variations by increasing the number of biological replicates, aiming for a sample size of n = 5 animals per group. Our bioinformatic analysis shows that we can detect several differentially expressed genes over a range of expression levels, also at small fold changes, highlighting the sensitivity of the protocol.

We chose to collect approximately 150 cells per cell population in order to represent the whole population rather than a subset of very heterogeneous motor neurons. We do acknowledge, however, that by analyzing bulk samples, we might lose biologically relevant information considering the vulnerability gradient within certain motor neuron pools. In SMA, spinal motor neurons of the medial motor column degenerate before lateral cells are lost. Within the levator auris longus (LAL) muscle, that receives input from the facial motor nucleus, the caudal band shows denervation whereas the rostral band appears to be resistant. However, employing single cell sequencing for our already complex study design (six differentially affected cell populations x three time points x two genotypes) seemed unfeasible. Nevertheless, we have gained important insights into SMA disease mechanisms that we can further interrogate on the single cell level in selected populations in future studies.

3.2.3 Full-length RNA sequencing: splicing analysis remains a challenge

Smart-seq2 uses oligo-d(T) primers and thus selects for poly(A)$^+$ transcripts but at the same time shows improved read coverage of the whole transcript length (gene body coverage) (Picelli et al. 2013). Sufficient gene body coverage is crucial for splicing analysis, which is particularly pertinent when studying SMA due to the function of the SMN protein in the assembly of the spliceosome.

We found a rather strong 3' bias of read coverage in our samples and initial splicing analysis led to the detection of only few significant splicing event changes, limited to the 3' end of the transcripts (**Figure 3.3**). Increased sequencing depth slightly improved the detection of splicing events over the whole transcript body when we performed analysis on pooled samples, but our results lacked biological significance when subsequently examining individual replicates. We therefore concluded that we cannot perform robust splicing analysis over the whole transcriptome for this study.

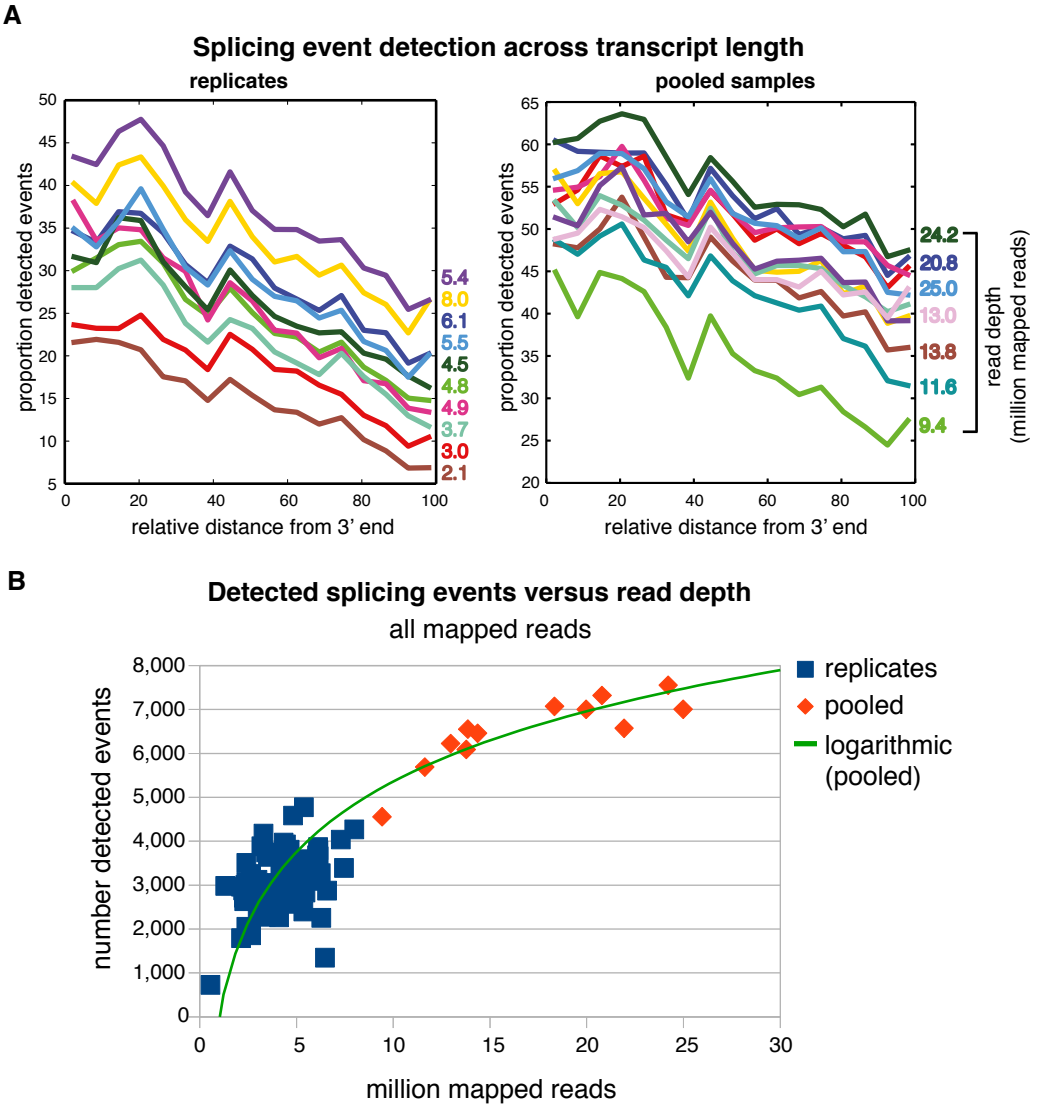


Figure 3.3. ***Splicing event detection improves with read depth but reveals differences in coverage along the transcript length.*** (A) Splicing analysis performed on individual samples revealed a constant decrease of detected splicing events from the 3' to the 5' end (exemplified for spinal cord samples). (B) Splicing analysis on pooled samples seemed to lead to a milder decrease in events detected towards the 5' end of the transcripts. (C) More splicing events were detected with increased sequencing depth. Colored numbers in A and B indicate the read depth of selected samples (million mapped reads).

The poor gene body coverage could be of many origins, the most obvious being RNA degradation that might occur during the several tissue processing steps. Preliminary analysis indicates that the RNA quality in our samples is compromised during the staining procedure for LCM. We prepared cDNA libraries from (1) stained tissue chunks and (2) tissue sections that had undergone the washing and dehydration steps (with ddH_2O and ethanol) while omitting the actual staining solution and compared them to (3) cDNA libraries obtained from unstained tissue chunks (air dried only) (**Figure 3.4**). cDNA profiles as measured with a Bioanalyzer (Agilent) visualize the fragment size distribution of a library and are good indicators of input RNA quality. cDNA libraries from tissue sections that were air dried only were of the highest quality, whereas unstained but H_2O- and ethanol- treated chunks resulted

in significantly better quality than samples stained with histogene staining solution. Sequencing data are necessary to better evaluate the gene body coverage obtained from the different tissue processing conditions. Nevertheless, we anticipate that by improving the staining procedure RNA degradation can be substantially decreased and thus gene body coverage increased.

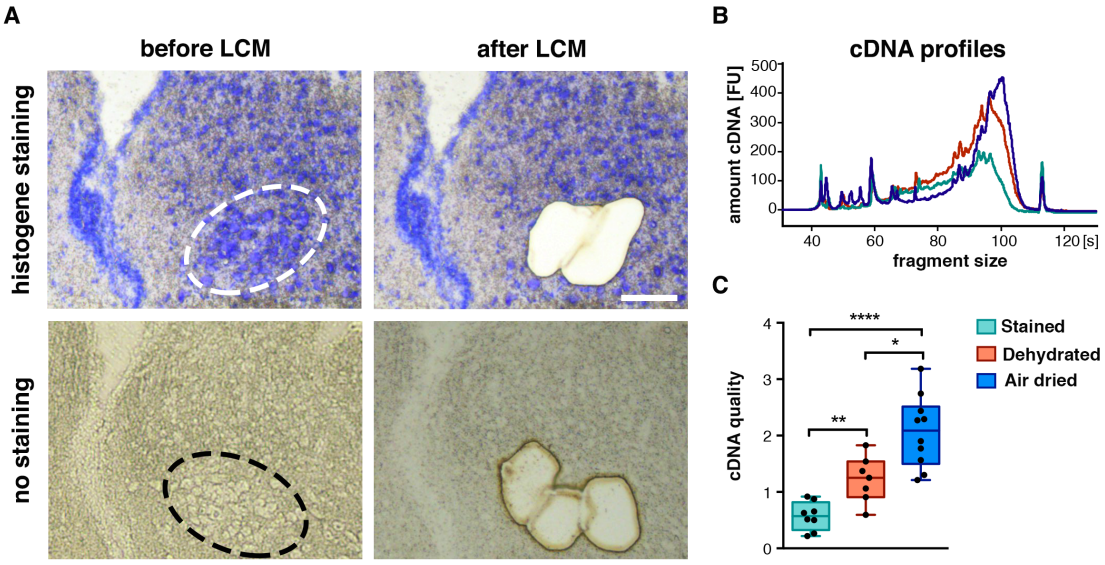


Figure 3.4. ***RNA quality is likely compromised during histogene staining procedure.*** (A) Examples of stained and unstained tissue sections before and after laser dissection of tissue chunks. Adjacent sections were used in order to ensure the inclusion of similar tissues. Scale bar in top right panel = 100 µm. (B) Examples of cDNA profiles that illustrate the difference between air dried (blue), unstained but H_2O- and ethanol- treated (red) and histogene stained (turquoise) tissue chunks. Fragment size is shown as migration time in seconds (s). The peak at 60 s corresponds to a fragment size of approximately 300 base pairs (bp) in all samples. Main upper peaks correspond to: ~ 900 bp (stained; turqouise), ~ 1500 bp (H_2O/ethanol; red) and ~ 2000 bp (air dried; blue). (C) Input RNA quality was estimated by calculating the ratio of the area under the curve between 600 – 3000 bp (range of mammalian RNA (Lewin 2008)) and 100 – 600 bp, and shows that RNA from tissue chunks that were air-dried only was best preserved, while the small ratio for histogene stained samples suggest RNA degradation. Boxplots with individual values and whiskers representing minimum and maximum. One-way ANOVA was performed with GraphPad Prism6, F (2, 22) = 21.90, Tukey's multiple comparison test; * = P<0.05, ** = P<0.01, **** = P<0.0001.

Another possible source of incomplete gene body coverage is inefficient reverse transcription (RT) of RNA species. RT enzymes have to overcome secondary structures in the RNA making RT along the full transcript length a challenge. Furthermore, performing this reaction directly in cell lysates as is the case for the Smart-seq2 protocol might have the presence of inhibitory factors as a consequence. Indeed, in paper III, we observed a slightly better gene body coverage for samples that had undergone RNA extraction before cDNA preparation indicative of more efficient RT in purified samples (**Figure 3.5**). For the development of the Smart-seq2 protocol several RT enzymes, buffer compositions and reaction conditions have been compared. In the meanwhile, new, improved RT kits are available from several providers and it will be worthwhile to test the latest protocols on LCM dissected cells.

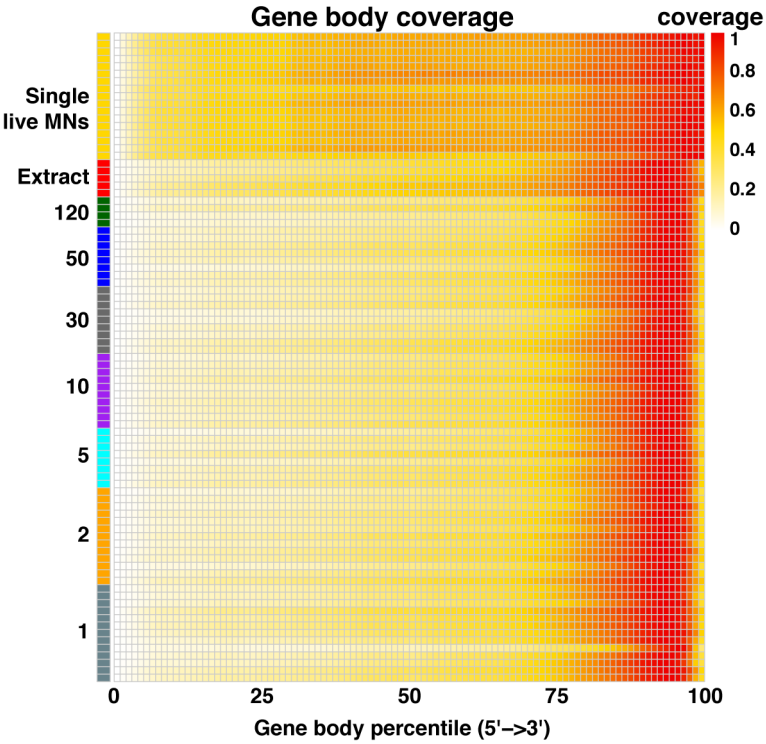


Figure 3.5. ***Compromised gene body coverage in LCM dissected motor neurons compared to live single motor neurons.*** Exemplified for transcripts that are shorter than 3 kilo bases. Samples that had undergone RNA extraction prior to cDNA preparation (Extract) displayed slightly improved coverage compared to samples where reverse transcription was performed in cell lysates. Numbers on the vertical axis indicate the number of cells collected. MN = motor neuron.

In summary, we are optimistic that by adjusting different steps in the protocol we will ultimately be able to achieve sufficient gene body coverage to perform also robust splicing analysis using this method. Nevertheless, we demonstrate here that we can obtain important insight into gene expression level changes in closely related neuronal populations using LCM-seq as discussed in paper III and IV. While we cannot entirely exclude the possibility that some transcripts detected originate from contaminating glial cells, the expression of multiple genes identified in study IV could be confirmed in motor neurons using the extensive *in situ* database provided by the Allen Institute for Brain Research (Lein et al. 2007). Further investigation via *in situ* hybridization and immunohistochemistry on healthy and SMA tissues will refine the candidate list for functional studies in *in vitro* and *in vivo* models of SMA as demonstrated in paper I and II.

4 RESULTS AND DISCUSSION

We are investigating cell intrinsic mechanisms that render some motor neurons, including oculomotor (CN3) and spinal motor neurons, resistant or vulnerable to degeneration in ALS and SMA. We hypothesize that we can use this differential vulnerability as a tool to identify drug targets for the treatment of human motor neuron diseases. In this thesis, we could demonstrate a distinct protein signature of different motor neuron populations that is relatively conserved in mouse and man. Moreover, we validated oculomotor resistance as a tool to identify candidates that can protect vulnerable motor neurons *in vitro* and *in vivo*. We have furthermore developed a protocol for robust RNA sequencing of LCM dissected cells (LCM-seq), that can aid in further elucidating transcriptional adaptations in closely related neuronal populations. We applied LCM-seq to explore disease mechanisms in a mouse model of SMA and were able to dissect shared and cell type specific transcriptional changes that provide insight into differential vulnerability in this motor neuron disease. In this section, I will describe the most important findings of each study in more detail.

4.1 Paper I. Motor neurons with differential vulnerability to degeneration show distinct protein signatures in health and ALS

Through a previous transcriptome analysis in the normal healthy rat, we could identify several transcripts that are preferential to resistant oculomotor neurons or vulnerable spinal and hypoglossal motor neurons and could thus underlie their differential susceptibility to degeneration in MNDs (Hedlund et al. 2010). Based on their function, we have chosen promising candidates (**Table 4.1**) that we here further investigated in the SOD^{G93A} mouse model of ALS and in human post-mortem patient tissues. In this study, we used immunohistochemistry to examine the expression of our candidates on the protein level and how they change in disease.

Table 4.1. Candidate genes selected for cross species analysis in health and ALS based on differential mRNA expression.

Gene name	Known biological functions	Motor neurons with highest mRNA level*
		Control rat
Gucy1a3	Main receptor for nitric oxide (Zabel et al., 1998), potentially implicated in modulating Fas ligand activation (Hedlund et al., 2010)	CNIII
Parvalbumin	Calcium-binding protein with motor neuron protective effects (Van Den Bosch et al., 2002; Dekkers et al., 2004)	CNIII
Gabra1	Inhibitory synaptic transmission, neuronal excitability (Lorenzo et al., 2006; Brockington et al., 2013)	CNIII
Gabra2	Inhibitory synaptic transmission, neuronal excitability (Lorenzo et al., 2006; Brockington et al., 2013)	SC
Peripherin	Intermediate neurofilament, over-expression of which causes ALS-like MN loss (Beaulieu et al., 1999)	CNXII and SC
Dynein	Retrograde transport protein, mutations in which are linked to motor neuron degeneration (LaMonte et al., 2002; Hafezparast et al., 2003)	CNXII and SC

CNIII = oculomotor nucleus, CNXII = hypoglossal nucleus, SC = spinal cord.
* Based on our previously published gene array study (Hedlund et al., 2010).

(references can be found in the corresponding section of Paper I)

4.1.1 Proteins with implications for motor neuron vulnerability and resistance are regulated in ALS

We have shown that several proteins with implications for motor neuron resistance, including $GABA_A$ receptor α1, guanylate cyclase soluble subunit alpha-3 and parvalbumin, were persistently expressed in oculomotor neurons in control (**Table 4.2**). Proteins with implications for vulnerability like dynein, peripherin, and $GABA_A$ receptor α2, which play roles in retrograde transport and excitability, and could place motor neurons at an increased risk, were found at higher levels in vulnerable motor neurons.

Table 4.2. Summary of candidate protein analysis across species in health and ALS.

Protein	Motor neurons with highest protein level			Changes in protein levels in disease
	Control mouse	Control human patient	End stage ALS human patient	Symptomatic fALS mouse
Gucy1a3	CNIII	CNIII	All motor neurons	Yes
Parvalbumin	CNIII	SC	CNIII	Yes
Gabra1	CNIII	CNIII	CNIII	Yes
Gabra2	SC	SC	SC	No
Peripherin	SC	CNXII and SC	CNXII and SC	Yes
Dynein	SC	CNXII and SC	All motor neurons	Yes

CNIII = oculomotor nucleus, CNXII = hypoglossal nucleus, SC = spinal cord.

With the exception of Gabra2, all candidates investigated here were modulated when challenged with a disease-causing mutation underlining their importance for normal motor neuron function. As their regulation could either be beneficial or detrimental, it could be causal or an effect of the disease, functional studies in *in vitro* and *in vivo* models of ALS are crucial in order to evaluate the nature of their regulation. Nevertheless, these proteins constitute important pieces of a so far unsolved puzzle that can help us to understand the basis of differential neuronal susceptibility in ALS.

4.1.2 Conservation of the protein signature in mouse and man

Importantly, the expression patterns of our candidates were highly similar in mouse and human supporting their relevance also for the human disease (**Table 4.2**). The relative conservation of signals across species infers that SOD^{G93A} ALS mice could be used to predict mechanisms of neuronal vulnerability in man.

4.2 Paper II. Differential neuronal vulnerability identifies IGF-2 as a protective factor in ALS

In paper II, we have further investigated the potentially protective role of the CN3/4 restricted neurotrophic factor insulin-like growth factor 2 (IGF-2) as IGFs are known motor neuron survival factors (Silva et al. 2009; Eustache et al. 1994). Like in paper I, we initially examined the presence of IGF-2 protein with immunohistochemistry in symptomatic ALS mice and in human patients. To functionally evaluate its effect in ALS, we tested if IGF-2 could protect vulnerable spinal motor neurons in *in vitro* models of ALS-like toxicity and subsequently performed *in vivo* experiments in SOD^{G93A} ALS mice.

4.2.1 IGF-2 is preferential to oculomotor neurons in symptomatic ALS mice and in end-stage ALS patient tissues

In order to evaluate if IGF-2 could play a role in oculomotor resistance, we first investigated its protein expression in symptomatic ALS mice and human end-stage ALS tissues and found significantly higher levels in resistant oculomotor compared to vulnerable hypoglossal and spinal motor neurons (**Figure 4.1**). Furthermore, IGF-1 receptor, which mediates survival pathways upon IGF binding, was also preferentially expressed in oculomotor neurons as well as on extraocular muscle endplates. This indicates that IGF-2 could have a protective role in the extraocular system that is absent in vulnerable spinal motor units.

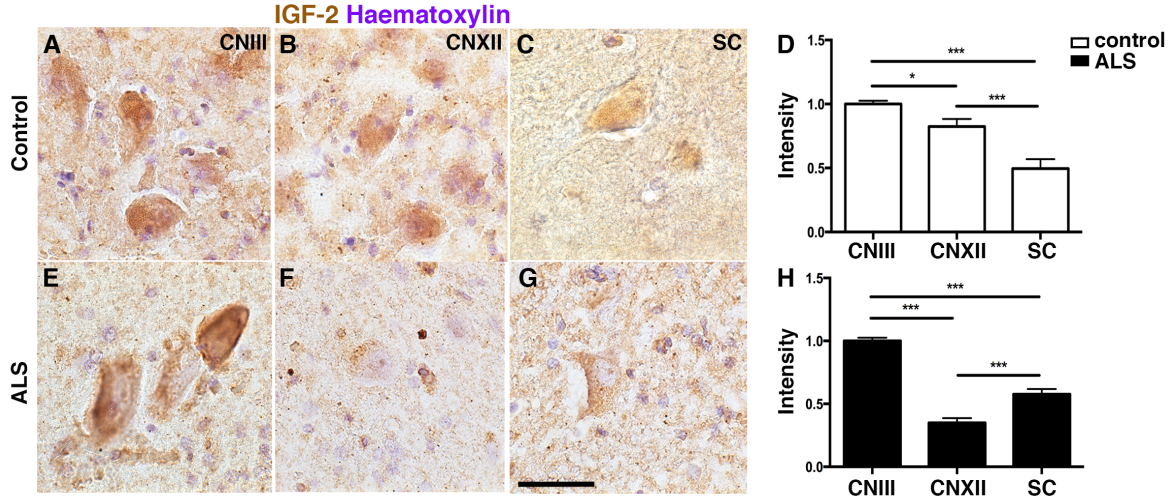


Figure 4.1. ***IGF-2 protein is persistently expressed in resistant oculomotor neurons in ALS end-stage tissues***. Immunohistochemistry was used to visualize IGF-2 protein expression in oculomotor (CNIII, A and E), hypoglossal (CNXII, B and F) and spinal (SC, C and G) motor neurons. In line with our results from the mouse tissue analysis, IGF-2 protein levels were highest in resistant oculomotor neurons in human control (A-D) and ALS patients (E-H). (D, H) Quantifications of staining intensity. One-way ANOVA with Bonferroni multiple comparison correction. *** = $P<0.001$, * = $P<0.05$. Scale bar in G applicable to A-C and E-G = 30 µm.

4.2.2 IGF-2 can protect vulnerable human motor neurons in vitro

To functionally support our hypothesis that oculomotor restricted factors could play a role in their resistance, we initially used two *in vitro* models of ALS-like toxicity to perform gain-of-function studies: glutamate- and mutant astrocyte- induced toxicity. Using iPSC-derived motor neurons from human ALS patients and controls, we showed that the addition of IGF-2 to the cell culture medium after initiation of toxicity significantly improved the survival of these vulnerable motor neurons in both assays (**Figure 4.2**). We furthermore demonstrated that the protective effect of IGF-2 is likely elicited by the activation of the PI3/Akt survival pathway as evidenced by the increased phosphorylation of Akt and GSK3β. The increased phosphorylation and thus inhibition of GSK3β is in line with a previous study that identified the GSK inhibitor kenpaullon as a survival factor for ALS patient- derived motor neurons (Yang et al. 2013).

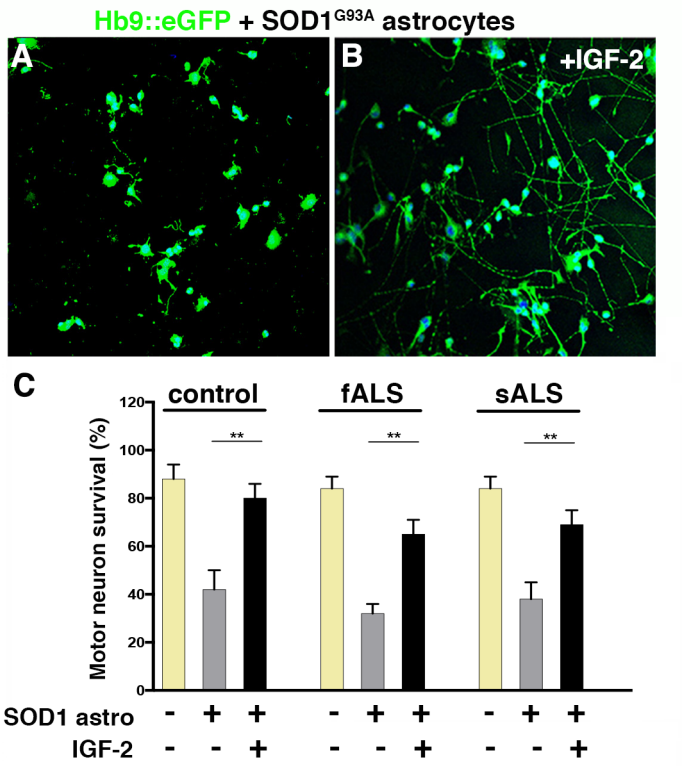


Figure 4.2. ***IGF-2 protects human iPSC-derived motor neurons from ALS-like toxicity.*** Shown here are the results from motor neuron cultures that were exposed to mutant astrocytes (A) without and (B) with the addition of IGF-2 to the cell culture medium. (C) Motor neuron survival was significantly increased by the addition of IGF-2. Values represent mean±SD from 5 independent experiments performed in triplicate. One-way ANOVA with post-hoc comparison, ** = $P<0.01$.

4.2.3 IGF-2 ameliorates disease in a mouse model of ALS

Due to the positive results in our *in vitro* experiments, we further asked if viral delivery of *IGF-2* to motor neurons could protect them in the SOD^{G93A} mouse model of ALS. We used an adeno-associated virus vector (AAV9) expressing *IGF-2* that was injected bilaterally into the hindlimb quadriceps and thoracic muscles at an early symptomatic stage. From the muscle, AAV9 is transported retrogradely to the soma of motor neurons to drive transgene expression. Compared to AAV9::null injected controls, the IGF-2 treated mice lived longer (on average 14 days) and had improved motor function as evaluated by rotarod. In treated mice that were sacrificed when the control reached end-stage, we found more motor neurons in the lumbar spinal cord and a higher axon density in the respective ventral roots. As

assessed by GAP-43 expression, a growth and regeneration marker (Verhaagen et al. 1988), we conclude that induction of a regenerative response in motor neurons of symptomatic mice could contribute to the amelioration of the disease phenotype (**Figure 4.3**).

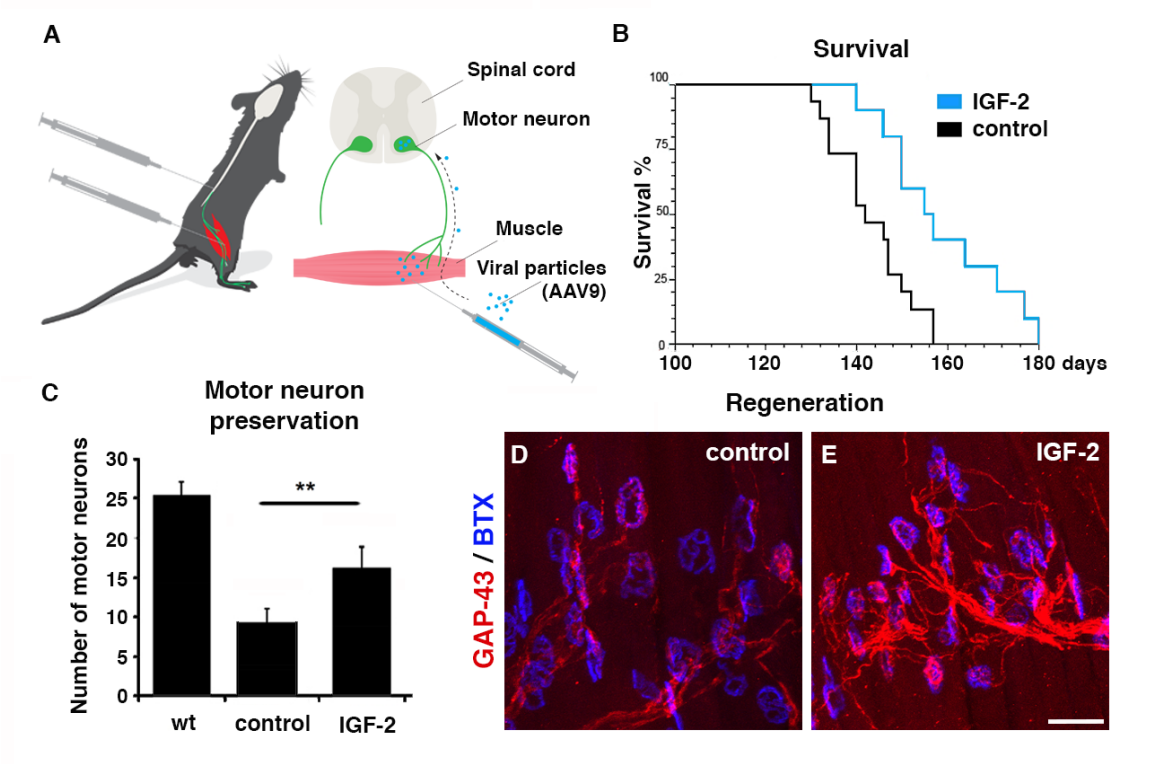


Figure 4.3. ***IGF-2 extends the survival of SOD^{G93A} mice by preserving motor neurons and inducing nerve regeneration.*** (A) Mice were injected at the age of 80 days with AAV9::IGF-2 or AAV9::null into hindlimb quadriceps and thoracic muscles from where viral particles are retrogradely transported to the spinal cord. (B) Survival of IGF-2 treated mice was significantly extended by 14 days (Kaplan- Meier survival curve, $\chi 2$ =5.3, P=0.02). (C) Motor neuron loss in IGF-2 treated mice was ameliorated. (D) GAP-43 staining of lumbrical NMJs indicated a regenerative response in IGF-2 treated mice. Control = AAV9::null injected mice, wt = wild-type untreated; One-way ANOVA with post-hoc comparison, ** = P<0.01. Scale bar in E, applicable to D and E = 40 µm.

Within the scope of this thesis, I would like to emphasize study II as a functional proof of concept for our approach. Here, we functionally demonstrated the protective effect of the oculomotor-restricted factor IGF-2 in vulnerable spinal motor neurons. We acknowledge, however, that loss-of-function studies are necessary to determine if IGF-2 plays a role in conferring resistance to oculomotor neurons. This could be tested by abolishing IGF-2 signaling in oculomotor neurons via stereotactic injections of shRNA into the oculomotor nucleus to block the survival-mediating IGF1R signaling. A complimentary approach would be the use of an *in vitro* system that assesses the survival of mESC- or iPSC-derived oculomotor neurons. Both strategies would however make it impossible to assign the resulting effects to IGF-2 specifically, as IGF-1 has a higher affinity for this receptor. Nevertheless, we would gain further insight into the role of IGFs in oculomotor resistance.

4.3 Paper III. Laser capture microscopy coupled with Smart-seq2 for precise spatial transcriptomic profiling

RNA sequencing has increasingly become a method of choice for exploring gene expression in biological organisms including man and mouse. It presents a powerful tool to understand cellular diversity and dissect normal biological processes and disease mechanisms. Often target tissues are scarce, such as human patient tissues or small cellular populations, and thus protocols for limited amounts of input material are necessary. Single cell RNA sequencing has become more and more feasible and is being successfully applied to live cells. Where tissues do not allow for easy dissociation or unique genetic markers are lacking for isolation of target cells (for example by FACS) other means of cell isolation are needed. Laser capture microscopy (LCM) can be used to dissect individual cells while preserving their positional information and it can be combined with antibody stainings to identify cell types of interest. Previously, the number of cells required to perform RNA sequencing after LCM dissection has been rather large with hundreds if not thousands of cells. Here, we have developed LCM-seq that couples LCM with Smart-seq2, a protocol for single cell RNA sequencing, for robust and efficient transcriptomic profiling of neuronal populations from snap frozen mouse and human tissues.

4.3.1 LCM-seq is reproducible and sensitive down to single cells

We initially collected 120 mouse spinal motor neurons and in order to find the minimum amount of input material needed, subsequently scaled down the number to single LCM dissected cells. The Smart-seq2 protocol employs reverse transcription of RNA directly from lysed cells and thereby circumvents the extraction of RNA and thus loss of material. In this way, while reducing cost and time, we were able to successfully prepare cDNA and sequencing libraries from only few cells with high success rates: 62% of the single mouse motor neurons, 82% of two cell samples and 100% of the samples collected with five or more cells passed our quality control and were used for further analysis. We could demonstrate a high reproducibility in gene detection for bulk samples, whereas the more variable expression profiles of fewer cells on the one hand indicates higher technical variation but can partly be explained by their biological variability (**Figure 4.4**). Encouragingly, we detected a comparable number of genes in single LCM dissected cells and live motor neurons derived from mouse embryonic stem cells (mESCs).

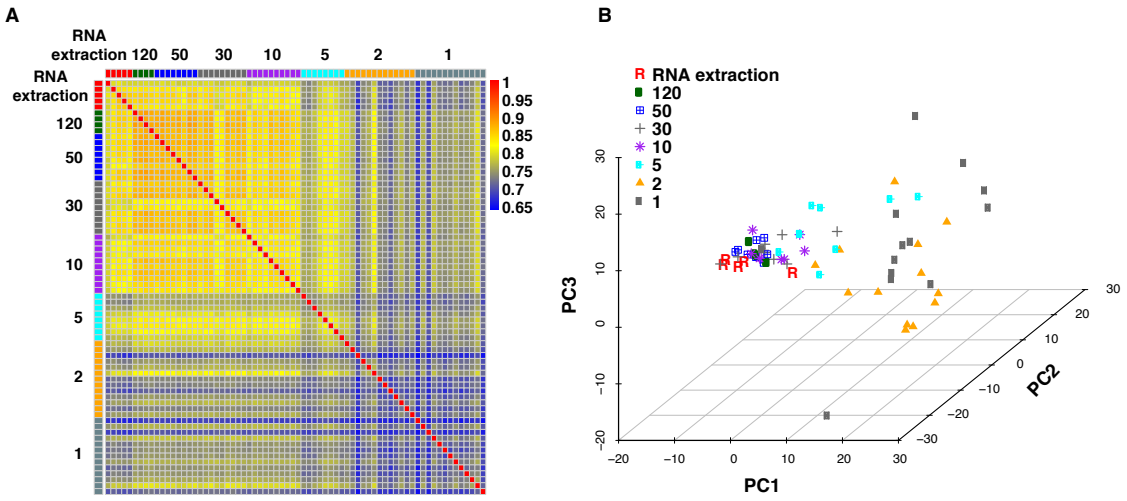


Figure 4.4. ***LCM-seq is robust and sensitive down to single cells.*** (A) Spearman correlation (including all genes expressed ≥1RPKM in at least one sample) and (B) principal component (PC) analysis of top 500 variable genes revealed highly related transcriptomes of samples with more than 10 cells. Lower correlation and larger spread of samples with 5, 2 and 1 cell reflects technical noise as well as biological heterogeneity of motor neurons.

As spinal motor neurons have a large soma and are metabolically active cells, they likely contain comparably large amounts of RNA. We therefore tested if LCM-seq is sensitive enough for single cell sequencing of smaller cells with presumably less RNA. The success rate for small neurons decreased substantially (16% of single cells and 33% of 5-cell samples passed the quality control). Thus, for cells of smaller size/RNA content it is important to increase the number of samples collected initially in order to obtain a sufficient sample size for analysis. Adjusting the staining protocol prior to LCM as discussed in the chapter '*Methodological considerations*' may prevent RNA degradation and improve the success rate. Yet another strategy could be to increase the number of amplification cycles during cDNA preparation. However, care must be taken as non-linear PCR amplification leads to the enrichment of highly abundant transcripts with the risk of losing low-abundance species. Nevertheless, in our study of small neurons, we could still detect an average of approximately 5,000 genes in one-cell samples and 7,000 genes in five-cell samples, making LCM-seq applicable to a variety of cell types.

4.3.2 LCM-seq can be used to distinguish closely related cell populations

We next tested if our method is sensitive enough to distinguish closely related cell populations, namely motor neurons isolated from different levels of the spinal cord. Using principal component analysis with the top 500 variable genes we found a clear separation of the two groups along the first component accounting for 51% of the variability in all samples. Among the top differentially expressed genes were numerous Hox transcription factors that confer positional identity during embryonic development. Besides serving as a positive control for distinguishing neurons derived from different positions along the anterior-posterior body axis, the persistent postnatal expression of Hox genes confirmed previous

results in the rat (Hedlund et al. 2010). In total, we identified 899 genes with differential expression in motor neurons of the cervical and lumbar spinal cord, demonstrating that LCM-seq can be used to dissect transcriptional differences in closely related cell populations.

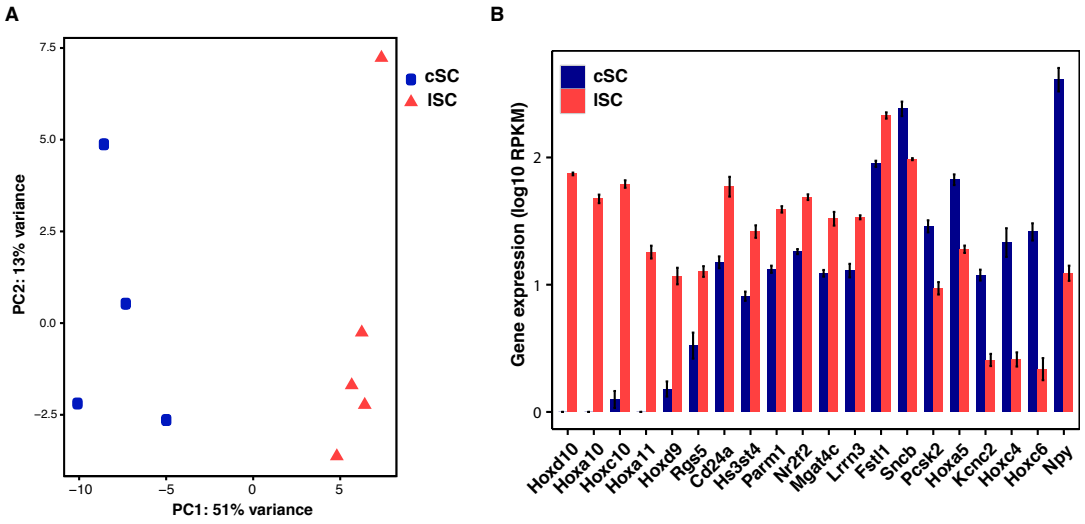


Figure 4.5. ***LCM-seq reveals distinct transcriptomes of closely related cell types that locate to different positions along the anterior- posterior body axis.*** (A) Motor neurons of the cervical (cSC) and lumbar (lSC) spinal cord separated clearly on the first component (PC1) in principal component analysis of the top 500 variable genes. (B) Among the top 20 differentially expressed were several Hox transcription factors that confer positional identity during embryonic development (shown as mean±SEM).

4.3.3 LCM-seq reveals transcriptomes of human neurons

Importantly, we could demonstrate that LCM-seq can be applied to human tissues that are partially degraded due to long processing times. We obtained robust sequencing data from midbrain dopamine neurons and spinal motor neurons with more than 10,000 genes expressed (≥1 RPKM) on average, in samples that contained less than 200 cells. Reassuringly, we found 4,903 differentially expressed genes between the two cell types.

Motor neurons can be easily identified by their large soma size and location and human dopamine neurons are characterized by their neuromelanin coat and thus a quick histological staining is sufficient prior to LCM. In order to test if our method could also be applied when identification of cells requires antibody staining with longer incubation times, we isolated dopamine neurons after staining with an anti-tyrosine hydroxylase antibody. Although the number of genes detected decreased compared to the histological staining (912 genes less, ≥ 0.1 RPKM), we could still detect 11,420 genes expressed at ≥ 1 RPKM, including dopamine neuron markers. Thus, LCM-seq can be applied to numerous cell populations that can be identified by antibody stainings. (**Figure 4.6**)

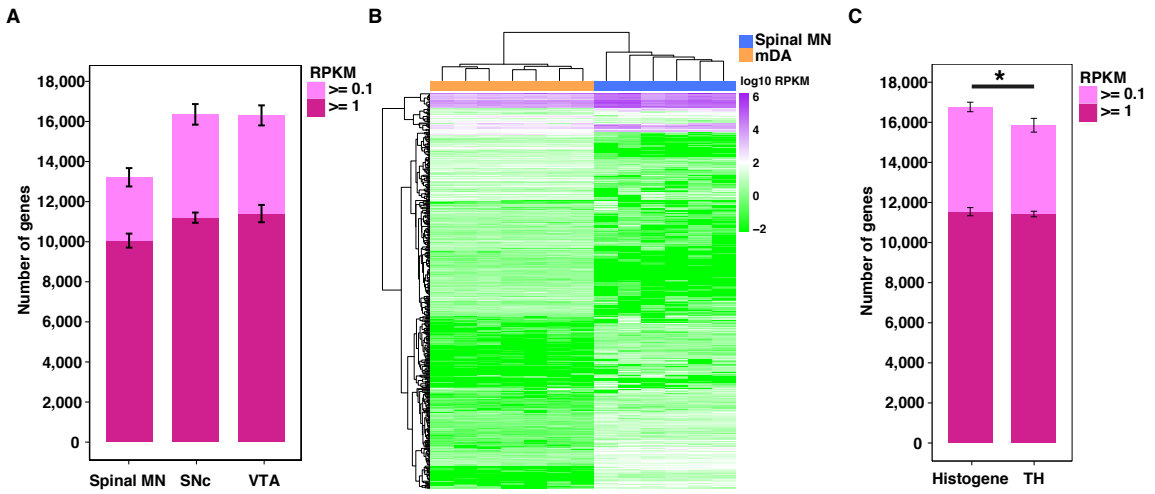


Figure 4.6. ***LCM-seq is applicable to partly degraded human post-mortem tissues and can be combined with antibody stainings.*** (A) On average, more than 10,000 genes (≥ 1 RPKM) were detected in spinal motor neurons (MN) and dopamine neurons of the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) (histological staining). (B) Clustering of midbrain dopamine neurons (mDA) and spinal MN based on the top 500 variable genes showed a clear separation of the two cell types. (C) A larger number of genes (≥ 0.1 RPKM cutoff) was detected when applying histological staining compared to antibody staining (Student's *t*-test, *P*=0.03). Values shown as mean±SEM.

Finally, we tested if LCM-seq can be successfully applied to single human motor neurons. Whereas mouse brains and spinal cord tissues were dissected in a few minutes (less than three and 15 minutes, respectively), processing times for human tissues used in this study were between three and ten hours likely resulting in increased RNA degradation. Nevertheless, we found an average of 7,655 genes expressed in these cells and could detect motor neuron markers like *MNX1* (Hb9), *ISLET1/2* and *CHAT* indicating that this method can be applied for single cell analysis of human tissues.

Taken together, we have developed a robust and reliable protocol for transcriptomic profiling of LCM dissected cells that can be applied to scarce neuronal populations and human post-mortem tissues. Our approach could thus be broadly used for studying general biological processes as well as human disease mechanisms.

4.4 Paper IV. Resistant and vulnerable motor neurons display distinct transcriptional regulation in spinal muscular atrophy

Spinal muscular atrophy (SMA) affects somatic motor neurons of the lower brainstem and spinal cord due to a deficiency in the broadly expressed survival of motor neuron (SMN) protein while oculomotor and trochlear (CN3/4) motor neurons are spared. In this study, we aimed for a comprehensive catalogue of transcriptional changes throughout disease progression in several differentially affected neuronal populations in order to better understand general disease mechanisms as well as neuronal susceptibility. We used LCM-seq to obtain gene expression profiles of vulnerable motor neurons (spinal cord (SC) and facial nucleus (CN7)) and of relatively resistant populations (oculomotor and trochlear (CN3/4) and hypoglossal (CN12) nuclei). We furthermore collected transcriptome data from visceral motor neurons of the vagus nerve (CN10) and neurons of the red nucleus (RN) that served as controls. (**Figure 4.7**)

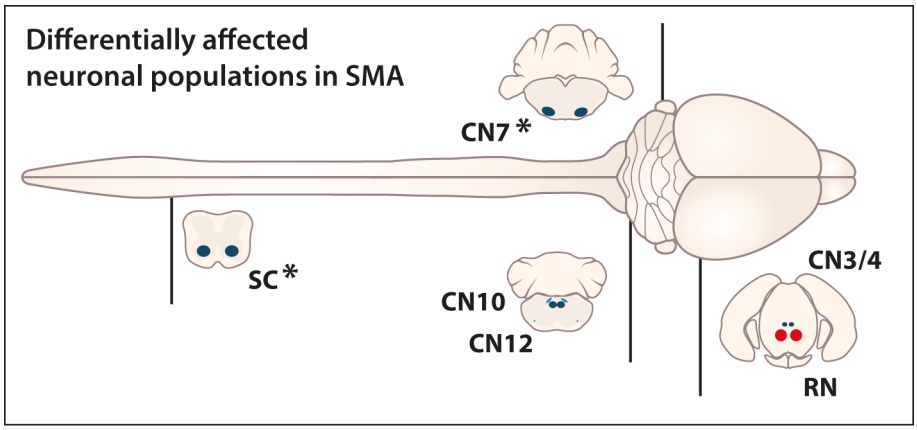


Figure 4.7. ***Cell populations with differential vulnerability have distinct locations in the brainstem and spinal cord.*** Lumbar spinal motor neurons (SC) are affected early in disease, whereas facial motor neurons (CN7) display pathology later (Murray et al. 2010). Hypoglossal (CN12) and oculomotor and trochlear (CN3/4) motor neurons are relatively resistant (Comley et al. 2016). Motor neurons of the vagus nerve (CN10) lie in close proximity to CN12 and serve as positional and importantly as a visceral motor neuron control that are unaffected in MNDs. Neurons of the red nucleus (RN) are located adjacent to CN3/4 motor neurons and share a developmental origin with these. They are unaffected in SMA and serve as a non-motor neuron control. Asterisks (*) highlight vulnerable populations.

4.4.1 Oculomotor neurons display a transcriptional profile that is distinct from other somatic motor neurons

Our initial analysis of the entire gene expression profile showed that the transcriptome of CN3/4 motor neurons is distinct from other somatic motor neurons confirming previous findings in mouse, rat and human (Kaplan et al. 2014; Hedlund et al. 2010; Brockington et al. 2013). Yet, CN3/4 motor neurons appear to be more closely related to somatic motor neurons than to visceral CN10 motor neurons which has not been disclosed in previous studies due to the lack of visceral motor neuron controls. (**Figure 4.8**)

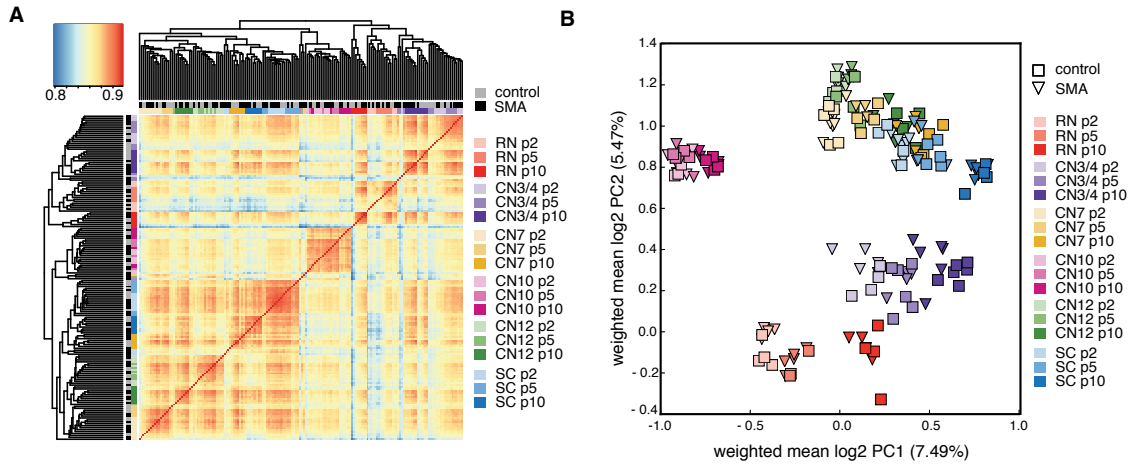


Figure 4.8. ***Distinct transcriptional profile of resistant CN3/4 motor neurons.*** (A) Spearman correlation showed that transcriptomes of somatic motor neurons of SC, CN7 and CN12 are highly correlated, whereas CN3/4 motor neurons clustered closer with RN neurons. (B) Principal component analysis of all genes expressed revealed that CN3/4 motor neurons are transcriptionally more similar to somatic motor neurons than visceral CN10 motor neurons. p = postnatal day.

4.4.2 Shared gene expression changes in somatic motor neurons imply DNA damage as a disease mechanism in SMA

Compared to our unaffected controls, RN and CN10, which both displayed regulation of 81 genes, we found substantially higher numbers of disease-induced differentially expressed genes in vulnerable as well as resistant somatic motor neurons (**Figure 4.9**). Comparable *SMN* mRNA levels in all groups investigated suggest that there is a qualitative difference in the need for SMN protein between cell types rather than a quantitative. We do acknowledge however, that the difference might lie in the efficiency to include exon 7 of the *SMN2* gene and thus produce full length transcripts and protein, which we cannot address with our study design as discussed earlier (section '*Methodological considerations'*). Motor neurons appear to have a low intrinsic efficiency to include exon 7 compared to other cell populations of the spinal cord. The primers specific for full length *SMN* mRNA derived from *SMN2* used in the study by Ruggiu et al. (2012) can be applied to investigate the efficiency of exon 7 inclusion in different neuronal populations including somatic motor neurons with differential vulnerability. Future investigations will thus shed light on this question.

While the overlap of differentially expressed genes was relatively low, suggesting cell type specific adaptation mechanisms, we could still identify common themes that were shared between all somatic motor neurons (**Figure 4.9B**). Most prominent was the activation of cell cycle, apoptosis and p53 pathway-related genes, including *Cdkn1a* (p21*)*, *Gtse1*, *Plk2*, *Fas*, *Pmaip1* and *Ptprv* that were differentially expressed in all somatic motor neurons. We also found these commonly regulated genes to be the strongest drivers of the genotype component in the principal component analysis of our most vulnerable (SC) and most resistant (CN3/4) groups. These findings are in line with several other SMA transcriptomics studies (Bäumer et al. 2009; Zhang et al. 2013; Maeda et al. 2014; Murray et al. 2015; Ng et al. 2015; Doktor et

al. 2016; Staropoli et al. 2015). P53 is a master regulator in response to several cellular stressors such as oxidative stress or DNA damage that can potentially lead to apoptosis. Even though the evidence for DNA damage in SMA is as yet limited, SMN could protect against transcriptional DNA damage through resolving R-loop formation (Zhao et al. 2015). FUS and TDP-43, which when mutated cause ALS, are involved in RNA regulation. As evidenced by increased DNA damage upon their depletion, FUS and TDP-43 contribute to the prevention or repair of transcription-associated DNA damage (Hill et al. 2016) and it appears that loss of SMN could have a similar result. Interestingly, a recent study demonstrated the presence of increased pH2AX, a marker for DNA repair complexes, in relatively resistant motor neurons of wild-type mice suggesting a higher intrinsic capacity for DNA damage repair. In resistant populations of SMA mice however, the marker was detectable at comparable levels to vulnerable motor neurons (Murray et al. 2015). Our analysis suggests that alternative protective mechanisms, potentially further downstream, could be at play. Within the p53 pathway, we found *Aldh4a1*, that can protect cells from oxidative stress (Yoon et al. 2004), and *Pak4*, a neuroprotective CDKN1A activated caspase inhibitor (Gnesutta et al. 2001; Gnesutta and Minden 2003; Won et al. 2016) to be up-regulated in CN3/4. Altogether, these findings suggest DNA damage as a disease mechanism in SMA and it will be interesting to disclose to what extent resistant and vulnerable populations are affected. Immunohistochemistry can be used to detect DNA damage markers like γH2AX (Mah et al. 2010) and BRCA1 (breast cancer 1, for transcription associated DNA damage (Hill et al. 2014, 2016)) in mouse tissues and will be applied to assess in which cell populations and at what time point DNA damage occurs in SMA and control mice.

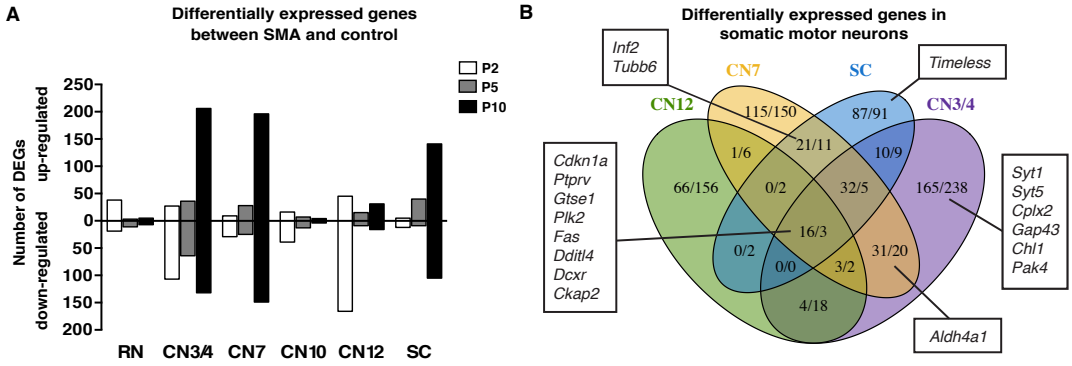


Figure 4.9. ***Resistant and vulnerable somatic motor neurons display distinct disease-induced transcriptional adaptation.*** (A) Somatic motor neurons regulated more genes in disease than unaffected control populations. (B) All somatic motor neurons displayed differential expression of genes related to DNA damage response and apoptosis pathways. Resistant CN3/4 neurons specifically up-regulated genes involved in synaptic transmission (*Syt1, Syt5, Cplx2*), regeneration (*Gap43, Chl1*) and up-regulated genes that protect from cellular stress (*Pak4, Aldh4a1*). Shared up-regulation of cytoskeleton-related transcripts (*Inf2, Tubb6*) in vulnerable SC and CN7 likely reflect axonal degeneration. Activation of *Timeless* expression could be a sign of exacerbated DNA damage in the most vulnerable SC motor neurons. P = postnatal day. No fold-change cutoff was applied and events with adjusted $P<0.05$ were considered significant. DEG = differentially expressed gene.

4.4.3 A vulnerability signature of spinal and facial motor neurons

In our analysis that compared spinal motor neurons and those of CN3/4 we identified *Timeless*, which determined SC identity and had a strong influence on the genotype component due to its drastic up-regulation in these neurons in SMA. *Timeless* is indirectly linked to the p53 pathway through its important role during DNA replication (Gotter et al. 2007) and it can furthermore mediate DNA damage repair after laser induced double strand breaks (Young et al. 2015). Along with the regulation of other numerous genes related to a DNA damage response, the strong up-regulation of *Timeless* only in SC could be a sign of exacerbated DNA damage in these cells. Both TIMELESS and FUS are recruited to sites of DNA damage in a PARP1- dependent manner (Rulten et al. 2014; Young et al. 2015) providing yet another link between mechanisms commonly activated in SMA and ALS.

Other genes, which separated *Smn1*- deficient spinal motor neurons from the most resistant CN3/4 motor neurons were tubulin beta 6 (*Tubb6*) and *Inf2*. Interestingly, these were also up-regulated in the vulnerable CN7 motor neurons. Altered expression of INF2, which is a potent actin filament severing protein (Gurel et al. 2014), and other cytoskeletal components at P10 is likely a sign of axon degeneration in these two vulnerable populations (**Figure 4.9B**).

4.4.4 Distinct SMA-induced transcriptional changes in resistant motor neurons

Using gene ontology (GO) term enrichment analysis, we observed terms like ‘nervous system development’ and ‘generation of neurons’ in our resistant groups. We speculate that the regulation of these genes reflects degenerative/regenerative responses. Several of these GO term genes up-regulated in CN3/4, including *Gap43* and *Chl1*, are also upregulated upon axon injury (Verhaagen et al. 1988; Zhang et al. 2000) supporting this notion. Moreover, it was recently shown that *Gap43* mRNA and protein levels were reduced in axons and growth cones of primary spinal motor neurons isolated from a severe mouse model of SMA (Fallini et al. 2016). This could imply a lower capacity for re-connectivity of vulnerable cells. Additionally, through our analysis of CN3/4 versus SC motor neurons, we found that *Syt1* and *Cplx2*, which are also in the nervous system development term, had a strong weight on the genotype and cell type component. SYT1 and CPLX2 function in neurotransmitter release, which appears to be impaired in SMA (Kariya et al. 2008; Ruiz and Tabares 2014). Furthermore, SYT1 has recently been associated with differential vulnerability in SMA (Tejero et al. 2016). Their CN3/4 specific up-regulation together with other genes associated with regeneration likely presents a protective mechanism that could contribute to their resistance in SMA (**Figure 4.9B**).

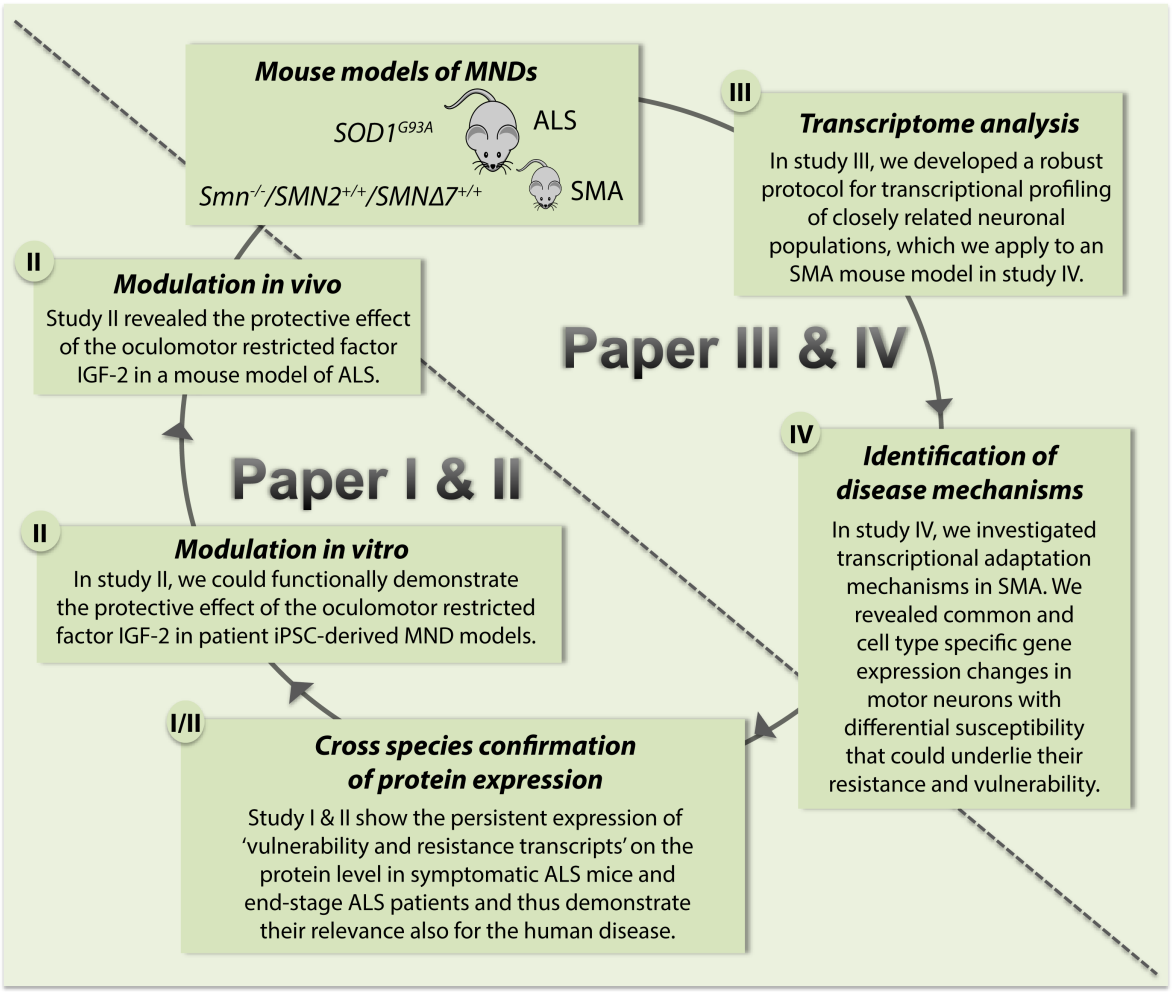
Investigation of the regenerative capacity of CN3/4 neurons will give us further insight into this hypothesis. Towards this goal, it will be interesting to investigate the presence of regeneration markers like GAP43 and CHL1 in the axons of these cells. Furthermore, axonal sprouting and the presence of growth cones can be easily assessed in

stained tissue sections. One has to keep in mind however, that such strategies may prove challenging due to the still ongoing remodeling of the NMJ during the first two postnatal weeks, which is likely accompanied by the aforementioned markers. The use of a milder SMA mouse model with longer survival is a possibility to circumvent these issues in such investigations.

Furthermore, we would like to point out that there is no clear consensus if CN3/4 motor neurons belong to the group of somatic or visceral motor neurons. Motor neurons of the ocular motor system are undoubtedly distinct from other somatic motor neurons in several aspects. They differ in their electrophysiological properties, underlined by the unique characteristics of the muscles they innervate. Their transcriptome furthermore diverges from those of spinal and hypoglossal motor neurons as shown in rat, mouse and man. Our transcriptome analysis adds important pieces to the puzzle by demonstrating that CN3/4 motor neurons are still closer to somatic motor neurons than visceral CN10 motor neurons. On the one hand, this is evidenced by higher correlation of the entire gene expression profiles in health. On the other hand, it is clear that adaptation mechanisms induced by SMN deficiency in CN3/4 are more similar to SC, CN7 and CN12 than to CN10 motor neurons. These findings furthermore highlight the value of using CN3/4 motor neurons as means of dissecting protective mechanisms that could be used to rescue vulnerable populations.

Taken together, future studies evaluating gain- and loss-of-function of selected candidates are necessary that will provide further insight into their role in vulnerability and resistance. Through our transcriptome analysis of neuronal populations with differential vulnerability in SMA, we could dissect disease-induced changes that are common to all somatic motor neurons and thus aid in understanding general disease mechanisms. Moreover, we have identified cell type specific, potentially protective gene expression changes that might underlie the differential vulnerability of somatic motor neurons and could eventually lead to the development of therapeutic targets.

Identification of Pathways of Degeneration and Protection in Motor Neuron Diseases



5 CONCLUSION

Motor neuron diseases preferentially affect specific neuronal populations with distinct clinical features even if disease-causing genes are expressed across many cell types and tissues. In SMA, somatic motor neurons are selectively vulnerable to the loss of the broadly expressed *SMN1* gene. In ALS, mutations in multiple ubiquitously expressed genes have been identified that result in the same selective vulnerability of somatic motor neurons. However, certain somatic motor neuron groups, including oculomotor and trochlear neurons, are for unknown reasons relatively resistant to degeneration. We hypothesized that we could use oculomotor resistance as a tool to dissect mechanisms of vulnerability and protection, which would aid in identifying drug targets for the treatment of so far incurable motor neuron diseases. The work presented in this thesis underlines the potential of this approach in several aspects:

In paper I and II, we further investigated candidates with implications for differential vulnerability that have been identified in a previous transcriptome analysis comparing resistant and vulnerable motor neurons in the normal rat. Importantly, we demonstrated a relative conservation across species by confirming the expression patterns of multiple proteins in mouse and human and in health and disease. Moreover, we provide functional evidence that the oculomotor restricted factor IGF-2 can rescue vulnerable spinal motor neurons thus indicating that it could in part play a role in oculomotor preservation in ALS. To further pinpoint protective and detrimental mechanisms, it is important to understand how cell populations with differential vulnerability adapt to the same toxic genetic changes. In study III, we provide a robust and sensitive protocol for transcriptional profiling of closely related cell types that we applied in study IV in order to dissect disease mechanisms in six different neuronal populations in SMA. Our findings here highlight the value of using a closely related but resistant population as a control to further dissect general disease mechanisms from adaptations that could confer susceptibility and resistance.

Taken together, we here provide a tool box for transcriptional profiling of neuronal populations with differential vulnerability followed by functional studies in mouse and human that could aid in elucidating pathological mechanisms in neurodegenerative diseases.

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