Using the natural resistance of motor neuron subpopulations to identify therapeutic targets in amyotrophic lateral sclerosis

Krista Spiller

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy under the Executive Committee of the Graduate School of Arts and Sciences

COLUMBIA UNIVERSITY

2014
ABSTRACT

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Though mutant proteins are broadly expressed in neurodegenerative diseases, only some neuronal subsets are vulnerable. In patients with amyotrophic lateral sclerosis (ALS), most motor neurons degenerate but those innervating extraocular muscles, pelvic sphincters and slow limb muscles exhibit selective resistance. Previous work in our lab used laser capture microdissection followed by microarray analysis of vulnerable lumbar level (L5) motor neurons and the resistant motor neuron populations that innervate the extraocular muscles (III) and the pelvic sphincter muscles (DL) to identify hundreds of molecular markers of each subset. By looking at the genes that had >10 fold differential expression and that were selectively and strongly expressed in motor neurons into adulthood, we identified matrix metalloproteinase-9 (MMP-9) as a potential susceptibility gene. We first examined the expression of MMP-9 in wild-type mice and found that it is expressed only after postnatal day 5 in fast motor neurons, which are selectively vulnerable in ALS. Further, MMP-9 was the only secreted member of the MMP family constitutively expressed in this population. In mutant SOD1 mice, MMP-9 expression was strongly positively correlated with cell loss at end-stage of the disease and with early induction of endoplasmic reticulum stress, as measured by p-EIF2α.

To test whether MMP-9 is a driver of disease, we crossed Mmp9 null mice with mSOD1 mice and found that both partial reduction and complete ablation of MMP-9 levels delayed muscle denervation, prolonged survival, and improved motor function, measured both behaviorally and electrophysiologically. Importantly, even acute knock-down in motor neurons
with AAV6 gene therapy or central inhibition of MMP-9 after symptom-onset were able to protect muscle innervation of the fast hindlimb muscle, TA. Further, MMP-9 expressed by motor neurons is required for full ER stress activation, suggesting it may be a very early intermediate in the disease pathway triggered by mutant SOD1. However, virally introducing MMP-9 into resistant pools does not confer susceptibility, implying that there is an additional factor (or factors) also present in fast motor pools that is necessary to induce axonal die-back. These studies suggest that MMP-9 is a promising candidate therapeutic target for ALS. Importantly, these data support the study of neuronal diversity as a potential way to define novel therapeutic strategies for the treatment of neurodegenerative diseases.
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Acknowledgements

First and foremost, I would like to thank my Ph.D. advisor, Chris Henderson. It has been a great privilege to learn from him. The guidance and training that he has given me has been exceptional. I would also like to acknowledge my thesis committee, Serge Przedborski, Lloyd Greene, and Neil Shneider, for their help to structure this project and for valuable feedback. Special thanks to Pico Caroni, for agreeing to read this thesis and serve as an external examiner.

I am deeply indebted to Artem Kaplan, who trained me and has continued to be an excellent collaborator and friend. His contributions greatly shaped this project. I also want to thank two summer students whose hard work was an immense help: Shruthi Deivasigamani and Adam Geber.

Further, I have been very lucky to have outstanding colleagues in the Motor Neuron Center, who have made this a great scientific community. All the members of the Henderson Lab, past and present, have been vital to the completion of this thesis. I would especially like to thank Nen Pagano, Dominick Papandrea and Ginn Choe for their help maintaining the mouse colony. I thank Kevin Kanning and Alan Tenney for great scientific discussions and thoughtful criticism. Finally, Monika Jackowski and Mariela Flambury provided essential administrative support.

I would like to acknowledge my previous mentors, whose guidance prepared me for graduate school: Eliot Gardner and Zheng-Xiong Xi (NIDA) and Allen Schneider (Swarthmore College).

A deep thanks to my friends from graduate school for valuable scientific insights. Thanks also to my friends who are not scientists, but can now speak fluently about selective motor neuron degeneration: Rhiannon Graybill, Alissa Shapiro, and especially Michael Silberfarb.

I want to acknowledge my large, wonderful family, whose support for me has never wavered. My parents in particular have given me everything and taught me to work hard, doing a job you truly love. My mother always emphasized the value of education and I never would have made it here without her. Special thanks to my sister, Klair, for a lifetime of good advice. Finally, I would like to offer my deepest gratitude to my twin sister, Kara Spiller, to whom this thesis is dedicated. People often joke about having a better half, but when I say it about her, I am not joking.
Chapter 1: General Introduction

For all neurodegenerative disorders, identifying mutant proteins in the familial forms of the disease has allowed researchers to make both cell culture and vertebrate models that have helped elucidate underlying molecular mechanisms of cellular dysfunction and eventual death. However, in all cases, the expression of these mutant forms of proteins leaves important unanswered questions with regard to the age-dependence and regional selectivity that characterize neurodegenerative diseases. Specifically, 1) what makes only some neuronal subsets vulnerable when mutant proteins are broadly expressed? And, 2) why do neurodegenerative diseases occur in middle age and old age despite the fact that mutant proteins are expressed over a patient’s whole lifetime? In this thesis, I will attempt to probe these questions through a study of regional selectivity in amyotrophic lateral sclerosis.

Part I. Selective neuronal loss is a hallmark of neurodegenerative disease

Selective vulnerability at the level of cell type, population, and subclass

The neurodegenerative diseases Parkinson’s disease (PD), Alzheimer’s disease (AD), and motor neuron diseases like amyotrophic lateral sclerosis (ALS) are estimated to affect some 20 million people worldwide (Mayeux, 2003). Though the clinical presentations of these three devastating disorders are very different, there are striking similarities between them. All three of these disorders feature onset that normally occurs during middle age or in the later years of life, each can occur as an inherited disorder but is more often sporadic, and all three are relentlessly progressive. Further, there is strong evidence that these disorders feature common underlying
cellular changes like protein misfolding and accumulation, mitochondrial dysfunction, oxidative stress, excitotoxicity, transcriptional dysregulation, neuroinflammation, and axonal transport defects (Double et al., 2010; Mattson and Magnus, 2006; Saxena and Caroni, 2011). A combination of some or all of these events impairs normal neuronal function and results in clinical signs that are often evident even before frank neuronal loss. However, as the diseases progress, dramatic differences between the clinical phenotypes of PD, AD, and ALS result from the remarkable degree of specificity in the identity of the neurons that degenerate in each and the resulting selective loss of the corresponding neuronal function. Put another way, at late disease stages, the symptoms are influenced more by the location of the cell death than the mechanism of cell death.

The first level of specificity of cell death is at the level of the neuronal class that is principally affected in these disorders (Figure 1.1A). For example, PD is the most common neurodegenerative movement disorder and is pathologically defined by dysfunction in basal ganglia circuits and the eventual selective cell death of dopamine neurons of the substantia nigra. AD is the most common form of dementia and the memory deficits associated with its onset result from a selective loss of cells in the entorhinal cortex, and CA1 and subiculum of the hippocampus. Finally, ALS is characterized by progressive paralysis that results from the cell death of upper and lower motor neurons.

However, for all of these disorders, there exists a second, finer-grained layer of selectivity: subpopulations within a single neuronal class. Using PD as an example, though dopamine neurons of the ventral midbrain are the class principally affected, subpopulations vary in their susceptibility to degeneration. Specifically, melanized dopamine neurons of the ventral tier of the substantia nigra (vtSN) are the most vulnerable, neurons of the dorsal tier of the
substantia nigra (dtSN) are more resistant and dopamine neurons of the ventral tegmental area (VTA) are the most resistant to degeneration (Elstner et al., 2011). This difference is apparent even though these cells form a continuous sheet of dopamine neurons that occupy the ventral midbrain and merge seamlessly with one another (Collier et al., 2011).

**Patterns of selective vulnerability in ALS**

ALS provides another clear example of regional selectivity at every level: though motor neurons are the class principally affected, individual motor pools at different levels of the neuraxis have very different disease vulnerabilities. Further, even within a given motor pool, there is a spectrum of disease susceptibility of different functional motor neuron subtypes. Like the other neurodegenerative diseases mentioned, ALS is age-dependent with clinical onset most commonly occurring between 40 and 60 years of age. Patients typically present with motor deficits in one particular region which result from the dieback of axons of motor neurons from individual muscle fibers and subsequent motor neuron death (Munsat et al., 1988). Early in the course of the disease, muscle denervation and motor neuron loss is compensated for by collateral reinnervation by the remaining motor neurons and this preserves strength (Bromberg et al., 1993). However, as the disease progresses, compensation fails and complete paralysis results when fewer than 5% of motor units remain (Swash and Schwartz, 1992).

The rate of loss of motor units is greatest at the site of onset of disease and the denervation of neuromuscular junctions has been shown to follow an exponential decline (Baumann et al., 2012). This is consistent with an early study which found that the motor neuron population in ALS patients is reduced by 50% every 6 months in the first year and more slowly
thereafter (Dantes and McComas, 1991). About one quarter of ALS patients exhibit primary bulbar symptoms, and there is a tendency for patients with bulbar onset to be older (mean 59 versus 55 years for limb onset) (Turner et al., 2012). Of non-bulbar patients, approximately equal numbers exhibited first symptoms in their arms (47.7%) or legs (46.5%), with 5.8% in both arms and legs, and, of these, the overwhelming majority presented in the most distal portions (73%) (Haverkamp et al., 1995). In fact, less than 3% of ALS patients present with motor deficits beginning in the respiratory or axial musculature (Shoesmith et al., 2007). Therefore, the motor neuron subsets that innervate respiratory muscles, which are part of the so-called “hypaxial motor column”, are much more spared by the disease than the motor pools which project to the limbs, those that comprise the “lateral motor columns” of the spinal cord (Kanning et al., 2010).

Importantly, even within a given motor column, nearby motor pools that innervate different muscles can have very different half-lives (Baumann et al., 2012), suggesting that individual motor pools have differing intrinsic susceptibility to disease. For example, in most ALS patients, the thenar hand is involved earlier and more severely than the hypothenar hand (Eisen and Krieger, 1993), a pattern of atrophy that is termed “split hand” (Wilbourn, 2000). Specifically, among the intrinsic hand muscles, wasting predominantly affects the median innervated muscles (abductor pollicis brevis and opponens pollicis) and the ulnar innervated muscles (first dorsal interosseous, adductor pollicis and flexor pollicis brevis), with relative sparing of the hypothenar muscles (the abductor digiti minimi)(Baumann et al., 2012). In fact, this is such a common divergence in weakness that over the past decade, the split hand has become increasingly recognized as a useful clinical sign of ALS (Figure 1.1C) (Eisen and Kuwabara, 2012). This example highlights that specific motor pools are differentially affected in
ALS, but a growing body of evidence reveals that even within a vulnerable motor pool, motor neuron subtypes are also differentially affected.
Figure 1.1
Figure 1.1 Specific regions and neuronal subclasses affected early in neurodegenerative diseases

A. There is a remarkable level of specificity in the identity of the first neurons that degenerate in each neurodegenerative disease (shown in red) and the corresponding neuronal function is what defines the clinical phenotype in these diseases. The hippocampus and some cortical areas are primarily affected in AD, the substantia nigra in PD, and the spinal cord and in ALS.

B. Dopamine neurons of the ventral midbrain vary in their susceptibility to degeneration, with neurons of the ventral tier of substantia nigra (vtSN, shown in red) being the most vulnerable, the dorsal tier of substantia nigra (dtSN, shown in yellow) being less vulnerable, and the adjacent cells of the ventral tegmental area (VTA, shown in green) being the least vulnerable. Figure is reproduced from (Collier et al., 2011).

C. Representative photo (left) and schematic (right) of an ALS patient’s hand showing prominent atrophy of the thenar eminence (red) but sparing of the hypothenar eminence (green). Photo is reproduced from Student BMJ archive

http://archive.student.bmj.com/issues/07/12/education/460.php [9/16/13]
Evidence for a role of motor neuron subtype in selective vulnerability

Each motor pool and the muscle it innervates is made up of a defined set of functionally distinct motor units, defined in 1925 as the “motoneurone axon and its adjunct muscle fibres” (Sherrington, 1925). In the 1970s, Burke and colleagues developed a classification scheme for motor units based on the physiological properties of the motor units (contractile time and sensitivity to fatigue) and the histochemical profiles of the associated muscle fibers (using a method of glycogen depletion for muscle unit identification) (Burke et al., 1971; Burke et al., 1973). They classified all motor units of the cat gastrocnemius as fitting into one of three major types, including two “fast” groups with relatively short twitch contraction times (types FF and FR, which were differentiable from one another on the basis of sensitivity to fatigue) and one “slow” group with relatively long contraction times (type S, which were extremely resistant to fatigue and were further distinguished from FF and FR units on the basis of the shape of unfused tetani in response to a standardized stimulus protocol) (Figure 1.2). Since the classification scheme was developed in those seminal papers, the three main functional types have been further differentiated by their recruitment order, their size and morphology, and their electrical properties (Kanning et al., 2010).

First, in a given motor pool, the earliest recruited motor units are type S; these exert the smallest forces and fatigue the least. Conversely, the last-recruited motor units are type FF, which exert greater forces but only for brief periods of activity (Duchateau and Enoka, 2011). The differences in size of the motor neuron subtypes and consequent electrical properties likely underlie this recruitment order (Kanning et al., 2010). Specifically, FF motor neurons are the largest subtype, followed by FR, and the S motor neurons are the smallest. But, S motor neurons are not just smaller than FF motor neurons; rather, they also show a clear difference in axonal
and dendritic branching complexity (Cullheim et al., 1987). The result of the size and differences in synaptic input is that smaller S motor neurons have higher input resistance and therefore require less synaptic activation to initiate action potentials. Accordingly, the large FF motor neurons are activated last. This rule is known as the “size principle” (Kanning et al., 2010; Mendell, 2005). Further, S type motor neurons were shown to have, on average, a significantly slower time course of post-spike after-hyperpolarization, a smaller rheobase, and more slowly conducting axons than those innervating fast-twitch muscle units (Bakels and Kernell, 1993). In reality, despite the appeal of these clear distinctions, it has been observed that the properties of the functional subtypes of motor units in both experimental animals and humans do not exhibit discrete distributions, but rather extend along a continuum from one extreme to another (Duchateau and Enoka, 2011).

There is also evidence that the functional subtypes of motor neurons are differentially susceptible to disease. One report from a human ALS patient who died as a result of surgical complications suggested there is early axonal dieback and selective susceptibility of FF axons (Fischer et al., 2004). However, most analyses are restricted to the postmortem examination of end-stage disease and cannot identify the earliest morphological changes in the motor unit. As a result, mouse models of ALS should be particularly useful in determining the order of motor unit death, provided they can be shown to faithfully mirror the disease process in humans.

Though most cases of ALS are sporadic, approximately 10% are classified as familial, or having a known genetic origin. Importantly, the pathology and pattern of selective motor neuron vulnerability is similar in familial and sporadic ALS (Shaw et al., 1997). Of the familial forms, the most studied and most common (roughly 20% of these cases) involve toxic gain-of-function mutations in the gene that codes for superoxide dismutase (SOD1). No common biophysical or
biochemical denominator has been found for all mutations in the small SOD1 gene (Andersen et al., 2003), but it is most commonly suggested that protein misfolding must be a common initial trigger (Boillée et al., 2006a). As I describe in more detail below, transgenic mice expressing mutant forms of human SOD1 (mSOD1) recapitulate the selective motor neuron degeneration and death seen in human sporadic and familial ALS (Chiu et al., 1995; Wong et al., 2002). This mouse model has allowed research to be done on the differential vulnerability of FF, FR, and S motor neuron subtypes.
Figure 1.2

A

B
Figure 1.2 Principle differences between alpha motor neuron subtypes

A. Alpha motor neurons innervate extrafusal muscle fibers to form three subtypes of motor unit: Slow-twitch (S) units control Type I fibers (yellow), fast-twitch fatigue-resistant (FR) units control Type IIa fibers (blue), and fast-twitch fatigable (FF) units control Type IIb/x fibers (green).

B. Different types of alpha motor neurons can innervate different fibers of a single muscle. The size and morphological complexity of alpha motor neurons diminish progressively from FF through FR to S motor units.
Early subtype-specific differences in disease susceptibility in mSOD1 mouse motor neurons

In the last 13 years, there have been several careful studies to elucidate the differential vulnerability of FF, FR, and S motor neuron subtypes. First, Frey et al. demonstrated that the functionality and maintenance of neuromuscular synapses are affected very early in motor neuron diseases, before any gross motor deficit is detected (Frey et al., 2000). The authors looked at muscle fiber subtypes in mouse triceps surae muscles (MGC, LGC, and soleus) and found that generally type IIb fibers (innervated by FF motor neurons) were located most laterally, and type I fibers (innervated by S motor neurons) were most medial. They also report that there was very little animal-to-animal variation in proportions of fiber types in these muscle compartments. Further, in the mSOD1 mouse, which typically begins showing gross motor dysfunction around 3 months of age, they reported extensive local muscle denervation already at postnatal day 50 (P50) in FF motor units. By P80, denervation and atrophy could also be observed in the medial gastrocnemius. However, in the slow soleus muscle, significant denervation was restricted to the very last phase of the disease (P120). Not only do the synapses on type I fibers resist denervation, they also sprout to reinnervate nearby vacated synapses (Frey et al., 2000).

A later study replicated this finding by observing neuromuscular junction denervation at P47, but also extended their analysis to include numbers of spinal motor neurons and axons in the nerve roots (Fischer et al., 2004). They found severe loss of motor axons from the ventral root between days 47 and 80, and loss of α-motor neuron cell bodies from the lumbar spinal cord after day 80. The authors thus suggested that the pattern implies that motor neuron disease in the mSOD1 mouse is a “dying back” motor neuropathy, where distal axonal degeneration occurs early during the disease.
Pun et al. went on to precisely characterize the remarkably synchronous timing of this dieback in FF, FR, and S motor units (Pun et al., 2006). Again in the mSOD1 model, they showed that there are two well-defined episodes of axon loss in the hindlimb muscles such as the tibialis anterior, lateral gastrocnemius, and soleus (Figure 1.3A). From P48-P52, muscles or parts of muscles innervated by FF motor neurons (such as the superficial portion of the tibialis anterior or the most lateral subcompartment of gastrocnemius) become completely and reproducibly denervated. In the portions of muscle innervated by a mix of FF and FR motor neurons (for example, in the deep, bone-facing third of the tibialis anterior), NMJs were partially denervated between P48 and P55, but were also partly reinnervated at P60 before a second denervation episode between P80 and P90 caused a permanent loss of NMJs and axons (Figure 1.3B). The slow soleus muscle showed no denervation or nerve loss before P85. The authors concluded that FF motor neurons disconnect their nerves from muscle first around P50, and then FR motor neurons innervating the same muscle sprout and reinnervate the unoccupied type IIb fibers. These new NMJs are unstable, and there is a second pruning phase around P80. Finally, type S motor neurons compensate efficiently through sprouting and those connections are maintained until the terminal disease stage. Electrophysiological and fiber typing studies have generally supported this general sequence of degeneration, though with subtle differences. Specifically, analysis of the TA muscle revealed a more gradual, incomplete loss of FF motor units accompanied by a parallel increase in FR motor units (Hegedus et al., 2007; Hegedus et al., 2008). However, the force of individual motor units was reduced, suggesting that the FR motor units were unlikely to have sprouted to reinnervate the vacant NMJs. Instead, the authors propose that a switch in motor unit phenotype from FF to FR precedes the apparent loss of FF motor axons (Hegedus et al., 2008).
These results have made it possible to investigate the process of disease-related axon loss with precision and identify earlier alterations in neuromuscular junctions and axons that are predictive of subsequent axon loss (Saxena and Caroni, 2007). For example, starting about 8–10 days prior to axon degeneration, vulnerable FF axons exhibit local accumulations of synaptic vesicles and a loss of synaptic vesicles from neuromuscular junctions, suggesting the presence of a defect in anterograde transport in these vulnerable axons (Pun et al., 2006).

Another change that precedes this subtype selective vesicular stalling and axonal dieback, is specific upregulation of the unfolded protein response (UPR) (Saxena et al., 2009). Using laser-capture microdissection of retrogradely-labeled resistant (soleus) or vulnerable (LGC) motor neurons of mSOD1mice, Saxena et al. they show that at early stages all mutant motor neurons transiently upregulate ubiquitin levels, likely reflecting ubiquitous expression of mutant SOD1 in the transgenic mice. However, only in the vulnerable (putative FF) motor neurons is there a concurrent upregulation of genes involved in stress-related pathways, including protein ubiquitination, hypoxia and NRF2-mediated stress. This stress-related response increased up to P26 but was abruptly lost at P32 and beyond. Further, the loss of early stress-related responses was accompanied by an upregulation of unfolded protein response (UPR)-related genes and a downregulation of ubiquitin proteasome system (UPS)-related genes in vulnerable motor neurons, a strong cellular stress response that peaked at P38. This pattern was further confirmed by immunocytochemistry for ATF4, PERK, and P-eIF2α. A similar wave of upregulation of UPR genes occurs in FR motor units later, and also appears to directly precede denervation. At P80, additional cell types besides just motor neurons also had upregulated UPR markers. Interestingly, treatment of mice with salubrinal, a blocker of dephosphorylation of eukaryotic
initiation factor 2α prevented upregulation of ER stress markers in FF motor neurons, delayed denervation of NMJs, and extended the lifespan of mSOD1 mice.

A very recent study extended these findings to include an analysis of MN excitability (Saxena et al., 2013). First, the authors used an antibody to detect disease-associated epitopes of SOD1 (misfSOD1) and found selective immunoreactivity in the FF subpopulation of α-MNs from P7. The misfSOD1 signal increased with age, eventually appearing in FR MNs before clinical disease onset (~P75 in their SOD1<sup>G93A</sup> “fast” mice). This protein misfolding colocalized in cells with an accumulation of ER stress signals (BiP and p-EIF2α) and was blocked with salubrinal treatment. In contrast, SOD1 misfolding and ER stress were potentiated with pharmacological treatment with compounds that reduced excitability (CNQX, Ketanserin + Way, and AP-5). Further, chronic CNQX treatment accelerated muscle denervation and motor dysfunction in these animals. These results demonstrated that reduced excitability was MN subtype-specific and resulted in aggregates and other disease-related pathology in mSOD1 mice.

Moreover, the authors also showed that enhanced excitability can protect vulnerable MNs (Saxena et al., 2013). AMPA treatment significantly reduced protein misfolding and ER stress and also delayed FR + S denervation (but not FF) and extended survival 25-30 days. Further, using a floxed pharmacologically selective module coupled to receptors for depolarization or hyperpolarization delivered unilaterally to lumbar α-MNs, the authors showed that this enhanced excitability is neuroprotective in a cell-autonomous manner. Finally, Saxena et al. implicated the mTOR signaling pathway, as SOD1<sup>G93A</sup> mice with treated with rapamycin had an exacerbated ALS phenotype whereas those treated with oxotremorine to stimulate mTOR and salubrinal to inhibit ER stress had significantly delayed MN degeneration and a lifespan extension of 50 days (2013). Therefore, these key findings suggest FF motor neurons are particularly vulnerable
because of their low excitability and moderate levels of glutamate receptor activation can engage adaptive cellular stress response pathways to promote MN survival.

Though these studies have greatly advanced our understanding of motor neuron subtype-specific susceptibility, the molecular basis of this selective FF-vulnerability remains an open question. Further, future studies on mechanisms underlying selective vulnerability would be greatly aided by having reliable molecular markers to identify these motor neurons subtypes beyond what are currently available (Kanning et al., 2010).
Figure 1.3

Muscles of the lower leg (lateral view)

Gastrocnemius

Peroneus longus

Soleus

Tibialis anterior

Achilles tendon

Extensor digitorum longus

B

% innervated

FF-innervation

FR-innervation

Age (days)
**Figure 1.3 Timing of axonal dieback by motor unit subtype in mSOD1 mice**

A. Muscles of the leg. Gastrocnemius, soleus, and tibialis anterior of the mutant SOD1 mouse were investigated to characterize the timing of axonal dieback in FF, FR, and S motor units. Picture is reproduced from http://www.nlm.nih.gov/medlineplus/ency/images/ency/fullsize/17238.jpg [9/17/13].

B. Timeline of the denervation of a typically FF-innervated muscle, reported by (Pun et al., 2006). The authors report a first pruning episode between P48 and P52 (dashed red line), followed by a period of reinnervation of the unoccupied type IIb fibers by the FR motor neurons innervating the same muscle at P60 (green dashed line). These new NMJs are unstable, and there is a second pruning phase around P80 (red dashed line).
A clear example of disease resistance: oculomotor and Onuf’s nuclei

Aside from the fast versus slow distinction with regard to differential vulnerability of motor neurons to ALS, several motor pools have been identified as being disease-resistant. In fact, two motor pools in particular are extremely well-preserved, even to late stages of the disease in patients: motor neurons innervating the extraocular muscles and those innervating the pelvic sphincter muscles (Kanning et al., 2010). The resistance of these motor pools (oculomotor, trochlear, and abducens nuclei in the midbrain and Onuf’s nucleus in the sacral spinal cord), corresponds to clinical studies that show a maintenance of motor function in ocular movement and voluntary control of eliminative functions (Mitumoto et al., 2006). In fact, it has been well documented that the cell bodies of these motor neurons are still intact at the time of ALS patient autopsy (Figure 1.4) (Iwata and Hirano, 1978; Kiernan and Hudson, 1993; Mannen et al., 1977; Okamoto et al., 1993; Schröder and Reske-Nielsen, 1984; Sobue et al., 1981).

Based on commonalities between these pools, there is a variety of potential mechanisms that could underlie their resistance to ALS. For example, like S motor neurons, oculomotor and Onuf’s motor units have small soma size. This could be relevant, as it has been speculated that disease vulnerability in motor neurons could result from the high energy demands and high metabolic rates that large cells with very long axonal processes have (Shaw and Eggett, 2000). That said, another way these resistant pools are different from other motor neurons is that they are almost always active, even during sleep (Enck and Vodusek, 2006; Navarro-López et al., 2004; Robinson, 1981), suggesting that metabolic demands alone are not enough to confer disease vulnerability. It could be the case that the oculomotor nucleus and Onuf’s nucleus are well adapted to dealing with the high energy requirements as it has been shown that these motor pools have greater calcium buffering capacity and express parvalbumin and calbindin (Alexianu
et al., 1994; Brockington et al., 2013; Shaw and Eggett, 2000). In fact, it has been reported that parvalbumin expression may contribute to a certain degree to ALS resistance, as the genetic overexpression of parvalbumin in neurons of mutant SOD1 mice resulted in attenuation of motor neuron loss by 33% and a modest survival increase of 11% (Beers et al., 2001). However, the expression data are somewhat unclear, as robust expression of parvalbumin has also been reported in vulnerable spinal motor neurons (Laslo et al., 2000; Sasaki et al., 2006).

Therefore, these groups have a number of potentially disease-resistant properties in common. Further, these extraocular muscle and pelvic muscle innervating motor pools have an advantage over fast and slow motor neuron subtypes in studying disease resistance because they are relatively easy to distinguish by location and morphology. Some molecular differences between oculomotor and other motor neurons have already been reported (Alexianu et al., 1994; Aronica et al., 2001; Hedlund et al., 2010; Laslo et al., 2000; Sasaki et al., 2006). For example, Hedlund et al. compared global gene expression profiles from laser-captured motor neurons of the oculomotor nucleus to those from the vulnerable hypoglossal nucleus and cervical motor neurons from adult rats and found that the oculomotor motor neurons had the most dissimilar transcriptomes (2010). Among the hundreds of genes differentially enriched in the oculomotor neurons, insulin-like growth factor II (Igf2) and guanine deaminase (Gda) were selected and shown to be protective in primary spinal cord cultures against glutamate-induced neurotoxicity. Though this study is very encouraging, the molecular basis of disease resistance in vivo still remains to be determined.
Figure 1.4

A

<table>
<thead>
<tr>
<th>Control</th>
<th>ALS</th>
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<tbody>
<tr>
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C

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Figure 1.4 Preservation of extraocular motor nuclei and Onuf’s nucleus in ALS

A. Images of midbrains at the level of the oculomotor nuclei from a control patient (left) and an ALS patient (right) stained with cresyl violet. Note preservation of large diameter purple-staining cells in the oculomotor nucleus of ALS patient.

B. Images of the ventral horns of lumbar spinal cord from a control patient (left) and an ALS patient (right) stained with cresyl violet. Note near complete absence of these cells in ALS ventral horn. Images in A and B are courtesy of Dr. Arthur Hays, Dept. of Pathology, Columbia University.

C. Images of the ventral horns of S2 spinal cord from a control patient (left) and an ALS patient (right) stained with Kluver-Barrera myelin stain. Onuf’s nucleus is located along the ventrolateral edge of the ventral horn is circled with a red dashed line. In S2 ventral horn of ALS patient, most LMC motor neurons have degenerated while Onuf’s nucleus appears virtually intact. Images are reproduced from (Mannen, 2000).
Part II. Shared gene expression in resistant motor nuclei compared to a vulnerable population

Given the clear distinction between the resistance of different populations of motor neurons, previous work in the Henderson lab focused on the extraocular and pelvic motor neurons of healthy mice in order to identify the molecular basis of their intrinsic disease resistance. Specifically, Artem Kaplan performed a comparative microarray to analyze gene expression in three groups of motor neurons: the resistant oculomotor and Onuf’s (DL in rodents) nuclei, and the disease vulnerable lateral motor column neurons of the L5 lumbar segment.

All three pools had some similarities as they all expressed a set of “generic motor neuron genes” (Figure 1.5A, yellow). However, the different motor populations also expressed a subset of genes linked to its specific function and rostrocaudal position. Further, an additional subset of genes were expressed in both resistant populations but were absent from vulnerable L5 motor neurons, and these were considered potential ALS resistance genes (Figure 1.5A, green). Conversely, those genes that were highly expressed in vulnerable L5 motor neurons but were absent from oculomotor and DL motor pools were considered putative ALS susceptibility genes (Figure 1.5A, red).

When the analysis was limited to genes whose expression levels showed >10-fold differences, only four genes were expressed at high levels in both resistant nuclei but at low levels in vulnerable L5, whereas 14 genes were more strongly expressed in vulnerable motor neurons than in the two resistant populations (Figure 1.5B). For half of these 18 genes, in situ hybridization was used to validate the microarray data. For example, Sema3E (semaphorin 3E) was confirmed to be strongly expressed in oculomotor and DL nuclei but absent from L5,
whereas $Mmp9$ (matrix metalloproteinase 9) and $Hsd17b2$ (estradiol 17-β-dehydrogenase 2) were strongly expressed in L5 motor neurons but absent from DL and oculomotor nuclei (Figure 1.5C).

Though limiting the analysis to >10 fold changes reduced the number of target genes (Figure 1.5B), further criteria were needed to select targets for further investigation. Because ALS is an adult-onset disease of motor neurons, the first criterion for selection was that a target gene be expressed selectively in motor neurons through adulthood. Of the group of potential disease susceptibility genes, only $Hsd17b2$ and $Mmp9$ maintained high levels at P57.

To choose between them, their ranking by three-way triangulation was compared, with the idea that a gene whose ranking was low in both individual pairwise comparisons but high using the three-way triangulation had the greatest chance of being linked to ALS rather than to specific properties of either pool. Table 1.1 shows the gene rankings by fold change, with the last column giving a number called the “added value of triangulation” (calculated by first ranking all of the genes from greatest fold change to least fold change, then taking the sum of the ranks in each 2-way comparison and subtracting from that the rank in the 3-way comparison). For example, MMP-9 was only 77th in the III/L5 list and 24th in the DL/L5 but was #10 in the 3-way comparison. Since $Hsd17b2$ was #3 on the comparison between DL and L5, $Mmp9$ was selected for further study. This decision was further supported by a broad existing literature on the multiple roles of MMP-9 in health and disease, as well as availability of reagents (reviewed below).
Figure 1.5

A

B

C

III

L5

L6 IC inj

Sema3e

Mmp9

Hsb17b2

RDL

DL
Figure 1.5 Microarray profiling of motor neurons from oculomotor (III), DL, and L5 motor neurons reveals robust genetic differences among these populations and points to a common set of genes relevant to ALS resistance

A. Experimental design. Comparison of genetic differences between mouse lumbar MN (L5), oculomotor nucleus (III, purple), and Onuf’s nucleus (DL, blue) provides markers of these populations among which genes related to ALS resistance may be found. ALS resistance genes are more likely to be found among genes commonly enriched (green) in both III and DL, while susceptibility genes (red) are more likely to be found among genes commonly depleted in III and DL.

B. Comparison of the numbers of differentially expressed genes with 10-, 7-, and 5-fold cutoffs. Note that regardless of the cutoff value, genes that are commonly regulated between III and DL as compared to the L5 motor neurons represent a much smaller set than comparisons among individual nuclei.

C. Sample validation of the microarray data. (top) ISH for Sema3e on cryosections of P7 brainstem and lumbar spinal cord. At L6, DL nucleus motor neurons are identified by injection of CT-B555 (red) into the IC muscle at P5. (middle and bottom) ISH for Mmp9 and Hsd17b2 on cryosections of wild-type brainstems and spinal cords at P7. Mmp9 and Hsd17b2 are expressed at different levels in subsets and L5 motor neurons, but oculomotor and DL nucleus motor neurons identified by injection of CT-B555 into the IC muscle at P5 do not express Mmp9 or Hsd17b2.
Table 1.1 Table of gene rankings by fold change

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Table 1.1 Table of gene rankings by fold change

The table shows the gene rankings of the top 20 potential susceptibility genes. Columns 2 and 3 show the fold changes for individual comparisons between DL and L5 and III and L5. Column 4 shows the rank of the genes with the greatest summed differences (calculated by summing the fold differences in the two pairwise comparisons). In column 7, we show the “added value of triangulation”, which is the sum of the ranks in each 2-way comparison minus that the rank in the 3-way comparison. Genes of particular interest are those not ranked very high in either pairwise comparison, but which come near the top of the list in the three-way comparison. By this reasoning, *Mmp9* (highlighted in yellow) was determined to be a very interesting candidate for further analysis.
Part III. Structure and function of MMP-9

MMP-9 is perhaps the most studied member of a large family of zinc-dependent proteolytic enzymes. In humans, 24 different MMPs have been identified (Lee and Murphy, 2004). These MMPs are generally divided into subgroups, based on their architectural features and, importantly, their preferred substrate or substrates. MMPs are grouped into gelatinases (MMP-2 and MMP-9), collagenases (MMP-1, -8, -13, and -18), stromelysins (MMP-3, -10, and -11), matrilysins (MMP-7 and MMP-26), membrane-type (MT)-MMPs that include 4 type I transmembrane proteins (MMP-14, -15, -16, and -24) and two glycosylphosphatidylinositol-anchored proteins (MMP-17 and -25), and 7 others that do not easily fit into a single category (Nagase et al., 2006). The main function of MMPs has been classically considered to be the degradation and removal of ECM molecules. However, recent reports have taken a more nuanced view of this role, emphasizing that the breakdown of ECM molecules or cell surface molecules alters cell–matrix and cell–cell interactions and the release of growth factors that are bound to the ECM makes them available for cell receptors (Cauwe et al., 2007; Nagase et al., 2006). Further, a number of non-ECM molecules are also potential substrates of MMPs and their importance in cell growth, the regulation of apoptosis, and cell migration are becoming increasingly appreciated (McCawley and Matrisian, 2001). Finally, though most MMPs are secreted and act extracellularly, some substrates that MMPs are known to process are localized intracellularly (Butler and Overall, 2009).

Structure of MMP-9

The activity of all MMPs is intimately linked to their structure, and MMP-9 serves as an excellent example of how the structure of these proteins affects their activity and substrate specificity. MMP-9 is secreted in a latent, zymogen form that requires posttranslational
modification to become activated. Specifically, MMP-9 consists of a propeptide domain, an active site, a zinc binding domain, a fibronectin domain, a linker (hinge) region, and a hemopexin domain (Figure 1.6).

The propeptide domain is made up of 80 amino acids at the N-terminus and consists of three α-helices and connecting loops. The loop between helix 1 and 2 is a protease-sensitive area, sometimes called a “bait region”, and a peptide region after helix 3 lies in the substrate-binding cleft of the catalytic domain (Vandooren et al., 2013b). This region contains the “cysteine switch”, which constitutes a fourth ligand of the active-site zinc, effectively blocking the active site and maintaining an inactive state (Visse and Nagase, 2003). MMP-9 is activated when a conformational change in the pro-domain pulls out the cysteine residue and enables water to interact with the zinc ion in the active site (Van Wart and Birkedal-Hansen, 1990). This change reduces the size of active MMP-9 to 82 kDa (from 92 kDa in the proform). The conformational changes that result in the disruption of the interaction between the cysteine switch and zinc will be discussed later in more detail.

The 3-D structure of the catalytic domain consists of a 5-stranded β-pleated sheet, three α-helices, and connective loops and contains one catalytic zinc, one structural zinc, and three calcium ions (Visse and Nagase, 2003). The substrate-binding cleft is formed by strand IV, helix B, and the extended loop region after helix B, and it contains a catalytically essential Glu402 and a Zn$^{2+}$ ion (Vandooren et al., 2013b). Between the active site and the metal binding site there are a cluster of three fibronectin repeats, and these are thought to be important for the binding and catalysis of large substrates such as elastins and denatured collagens or gelatins (Shipley et al., 1996; Vandooren et al., 2013b).
The fibronectin portion of the protein is connected to the C-terminal hemopexin domain via a flexible hinge region originally named the collagen type V-like domain, but now more accurately called the O-glycosylated domain. This hinge region consists of more than 50 amino acids, the longest linking region of all MMPs (Van den Steen et al., 2006) and this unusual length and the greater flexibility it provides to the molecule is the biggest structural difference between MMP-9 and the closely related MMP-2 (Rosenblum et al., 2007). Importantly, the O-glycosylated domain allows for independent movements of the terminal hemopexin and catalytic domains of the MMP-9 molecule (Vandooren et al., 2013b). It was also reported that a recombinant MMP-9 mutant with the O-glycosylated domain deleted showed decreased affinity to TIMP-1, low-density lipoprotein receptor related-protein-1 (LRP-1), and megalin, suggesting that this linking domain is essential for regulating the bioavailability of active MMP-9 (Rosenblum et al., 2007; Van den Steen et al., 2006).

The importance and uniqueness of the hemopexin domain of MMP-9 is also becoming increasingly appreciated. Whereas the prodomain and catalytic domains are very conserved between MMPs, the hemopexin domains are more differentiated (Andreini et al., 2004). Specifically, MMP-9 is the only secreted MMP that forms a homodimer through its hemopexin domain mainly by hydrophobic interactions (Cha et al., 2002) and that has a cysteine residue in this domain (Dufour et al., 2008; Van den Steen et al., 2006). In fact, because the hemopexin domain of MMP-9 shares such low amino acid identity with other secreted MMPs (only 25% to 33%), it has been proposed that more selective and specific inhibitors of MMP-9 could be developed by targeting this domain (Dufour et al., 2011). Moreover, its 3-D shape has been described as barrel-like, with a 4-bladed β-propeller structure with a single disulfide bond between the first and the fourth blades (Nagase et al., 2006).
Four major functions have thus far been ascribed to the hemopexin domain of MMP-9, including: interaction with substrates, binding to inhibitors, binding to cell surface receptors and induction of auto-activation (Vandooren et al., 2013b). The hemopexin domain has been reported to have a high affinity for substrates like collagen type I and IV, elastin, and fibrinogen. Interestingly, the hemopexin domain of the inactive proMMP-9 has been shown to have higher affinity for binding these ECM components than the hemopexin domain of active MMP-9, without the propeptide (Burg-Roderfeld et al., 2007). However, though interactions with the hemopexin domain appears to regulate the enzyme’s bioavailability, the actual cleavage of a number of substrates (including IL-8) by MMP-9 is equally efficient for an MMP-9 mutant without the hemopexin domain (Van den Steen et al., 2006).

Further, because dysregulation of the net activity level of MMP-9 is pathological in multiple situations in both the periphery and in the central nervous system, as reviewed in more detail below, another important role of the hemopexin domain is inhibition, which occurs directly and indirectly through interactions with endogenous inhibitors. First, MMP-9 activity has been shown to be specifically reduced by interaction with its own hemopexin domain, expressed in *E. coli* (Roeb et al., 2002). The authors speculate that the reason for this antagonism is because of the hemopexin domain’s affinity for gelatin; that is, the catalytically inactive hemopexin domain binds to the substrate and competes with active MMP-9. Second, besides this type of antagonism, the hemopexin domain of MMP-9 also asserts an inhibitory effect via its interaction with endogenous tissue inhibitors of metalloproteinases (TIMPs). There are four TIMPs (TIMP-1,-2,-3, and -4) which act as broad spectrum inhibitors of MMPs, though with differences in their specificity. Generally, the overall shape of the TIMP molecule is like a wedge, which fits into the active-site cleft of an MMP (Nagase et al., 2006). Though all TIMPs can inhibit MMP-9,
MMP-9’s hemopexin domain has specific interactions with TIMP-1 and TIMP-3 that increase the efficiency of inhibition. In fact, when the hemopexin domain of MMP-9 is deleted, TIMP-1 inhibits 17.5-fold less efficiently than it does the intact proteinase (Goldberg et al., 1992). Also, like TIMP-1, TIMP-3 can bind to proMMP9, but not to a mutant that lacks the hemopexin domain, indicating that these C-terminal domain interactions are sufficient and necessary to yield a stable proenzyme-inhibitor complex (Butler et al., 1999).

Another way in which MMP-9’s hemopexin domain is involved in the tight regulation of its activity levels is through its interaction with receptors at the cell surface. For example, the hemopexin domain interacts with LRP-1, and this interaction leads to endocytosis and intracellular degradation of MMP-9 (Hahn-Dantona et al., 2001; Van den Steen et al., 2006). Interestingly, the binding sites for LRP-1 and TIMP-1 in the hemopexin domain do not overlap, as the MMP-9/TIMP-1 complex still binds to LRP-1. This could suggest that these two routes of MMP-9 activity inhibition can occur simultaneously (Piccard et al., 2007). As an example of the potential importance of the interaction of LRP-1 and the hemopexin domain of MMP-9, LRP levels and activity are known to be substantially decreased in tumors (de Vries et al., 1996), which decreases catabolism of MMP-9 and could be one of the leading reasons why there are higher levels of MMP-9 at the tumor site (Hahn-Dantona et al., 2001). Aside from this interaction leading to endocytosis, the hemopexin domain of MMP-9 also interacts with docking molecules such as the Ku protein and CD44 to localize MMP-9 to cell surfaces, thereby facilitating their activation and proteolytic activities (Monferran et al., 2004; Stamenkovic, 2000).

Another potentially important role for the hemopexin domain of MMP-9 may be zymogen activation. Though auto-activation has been much more studied in other MMPs, particularly
MMP-2 and MMP-14 (Morrison and Overall, 2006), it has now also been reported for MMP-9. Specifically, it was shown that the interaction of hemin or β-hematin with the proMMP-9 hemopexin domain primes MMP-9 activation via an autocatalytic process (Geurts et al., 2008).

Finally, the hemopexin domain of MMP-9 also exerts non-catalytic effects through its binding with integrins. The binding of the MMP-9 hemopexin domain to α4β1-integrin (in conjunction with CD44v) contributes to B-cell leukemia lymphocytes pathogenesis in a way that does not require catalytic activity, but is simply mediated by the binding (Redondo-Muñoz et al., 2008; Redondo-Muñoz et al., 2010; Ugarte-Berzal et al., 2012). Further, when the interaction between the integrin epidermal growth factor (I-EGF)-like domains of β5 integrins and MMP-9’s hemopexin domain is blocked by a synthetic peptide, in vitro cell migration and in vivo tumor cell growth are prevented, without any direct effect on MMP-9’s proteolytic activity (Björklund et al., 2004).

Therefore, the relationship between the hemopexin domain and MMP-9’s net activity levels is a complicated one as the hemopexin domain has been shown to 1) inhibit activity, 2) activate proMMP9, and 3) have entirely independent effects from the enzymatic activity.
Figure 1.6

(A) Catalytic region:
- Signal peptide
- Propeptide
- Active site
- Fibronectin-like domain
- Zn$^{2+}$ binding site
- Hemopexin

92 kDa
**Figure 1.6 Schematic of the primary and 3D structure of MMP-9**

A. Domain structure of MMP-9 which consists of a signal peptide (white), a propeptide (green), a catalytic domain that contains the active site (red), a zinc ion (grey), and three tandem repeats of fibronectin type II inserts (blue), a proline-rich and heavily O-glycosylated linker (dashed line), and a hemopexin-like domain (grey).

B. Ternary structure of MMP-9, where the domains are shown in the same colors as in A. Figure modified from Dziembowska and Wlodarczyk (2012).
Regulation of the enzymatic activity of MMP-9

Given its large number of substrates and the potential destruction that unregulated proteolysis could cause in the body, MMP-9’s activity is tightly regulated at multiple levels (Figure 1.7). A first level of regulation is transcriptionally, and there are a variety of signaling pathways that stimulate or reduce the transcription of the Mmp9 gene. Next, MMP-9 is regulated at the level of the secretion of its proform (“proMMP9”). Importantly, once proMMP9 is secreted, it still must be activated. Finally, once activated, this activity is regulated by endogenous MMP inhibitors.

Like most MMPs, MMP-9 is not constitutively expressed in the majority of cells, but gene transcription is induced in response to stressors or other signals. In fact, there is a wide literature on its transcriptional control by cytokines, growth factors, hormones and other cell interactions (Opdenakker et al., 2001). The gene for MMP-9 is located on human chromosome 20 and its transcription is regulated by 670 base pairs of upstream sequence which includes binding sites for activator protein 1 (AP-1), nuclear factor kappa B (NF-κB), the Ets factor PEA-3, and specificity protein 1 (Sp-1) (Sato and Seiki, 1993). While Sp-1 and PEA-3 act as co-activators, the AP-1 and NF-κB binding sites appear to be essential for Mmp-9 gene expression. Correspondingly, the most often reported cause of suppression of MMP-9 transcription is interference with the binding of NF-κB and AP-1 to the promoter region (Vandooren et al., 2013b). For example, SIRT1 is a repressor of Mmp9 transcription and the inhibition of SIRT1 results in increased epigenetic expression of AP-1 and NF-κB by histone-4 acetylation (Nakamaru et al., 2009). It is also worth noting that resveratrol, a SIRT1-activating molecule, as well as lentiviral expression of SIRT1 in the hippocampus, slowed in vitro neuronal death as well as in vivo neurodegeneration and cognitive decline, though a connection with MMP-9 repression
was not investigated (Kim et al., 2007). Finally, another example of a compound that has been shown to repress \textit{Mmp9} transcription is Simvastatin, through a mechanism by which RhoA/ROCK inhibition leads to a reduction in \textit{Mmp9} mRNA levels (Turner et al., 2005).

Simvastatin has an additional effect on MMP-9 activity, in that it and another HMG-CoA reductase inhibitor, fluvastatin, also reduce secretion of proMMP9 from macrophages, an effect that can be overcome by exogenous addition of mevalonate, a precursor of isoprenoids (Bellosta et al., 1998).

Compartamentalization and secretion of MMP-9 has the most substantial effect on the timing of its activation. MMP-9 produced by neutrophils is stored in zymogen granules, ready to be secreted very quickly (<15 min) upon an inflammatory stimulus, without the need for transcription and translation (Van den Steen et al., 2002). This secretion has been shown to be mediated by Rab27a, a GTPase involved in specific vesicle trafficking (Brzezinska et al., 2008). In contrast, it was very recently demonstrated that in M1-type macrophages, MMP-9 can be found intracellularly in small Golgi-derived cytoplasmic vesicles along with calreticulin (CRT) and protein disulfide isomerase (PDI), and the secretion of these vesicles is not Rab27a dependent (Hanania et al., 2012). Also, because these macrophages rely on \textit{de novo} synthesis prior to the secretion of MMP-9, release takes several hours (Opdenakker et al., 2001). Though secretion is important, it is also worth mentioning that a broad range of intracellular proteins located in vesicles, mitochondria, the nucleus and cytoplasm can be processed by MMP-9 (Cauwe and Opdenakker, 2010), though there is some debate on whether that actually occurs intracellularly or if those proteins are also secreted and processed in the extracellular milieu.

As mentioned above, MMP-9 has an aminoterminal propeptide domain which contains the “cysteine switch” that is responsible for the latency of proMMP-9. Any means that can pull
the cysteine away from the Zn$^{2+}$ ion will result in activation and catalytic activity (Vandooren et al., 2013b). The earliest demonstrated in vivo activator was MMP-3 (Ogata et al., 1992), though even before that it had been documented that compounds, such as AMPA or trypsin could activate proMMP-9 in vitro (Masure et al., 1990; Moll et al., 1990). Since then, other activators have been identified including plasmin (Gong et al., 2008), kallikrein-related peptidase 7 (Ramani et al., 2011), MMP-26 (Yamamoto et al., 2004; Zhao et al., 2003), and human neutrophil elastase (HNE) (Jackson et al., 2010).

Perhaps of particular interest in a thesis about neuronal degeneration, proMMP9 can also be activated by nitric oxide (NO) by a process called S-nitrosylation. Gu et al. first showed that NO can directly activate proMMP9 and induce neuronal apoptosis in cerebrocortical cultures (Gu et al., 2002). Then, to confirm their results in vivo, they used mass spectrometry to characterize the events during focal ischemia and reperfusion. In this way, they showed that proMMP9 is activated by S-nitrosylation of a cysteine residue followed by a further irreversible modification of the residue into a sulfinic (−SO$_2$H) or sulfonic (−SO$_3$H) acid.

Once activated, MMP-9 is subject to a final level of activity regulation: inhibition, usually by TIMPs. Though all TIMPs can inhibit active MMP-9, TIMPs-1 and -3 have the best inhibitory efficacy. Further, most cell types that express MMP-9 also express TIMP-1 (Vandooren et al., 2013b). Chemical inhibitors have also been developed and have been used in various different avenues of research and these will be discussed in detail later in this chapter.
Figure 1.7
Figure 1.7 Schematic of the multiple layers of regulation of the activity of MMP-9

First, transcription is regulated by 670 base pairs of upstream sequence which includes binding sites for activator protein 1 (AP-1), nuclear factor kappa B (NF-κB), the Ets factor PEA-3, and specificity protein 1 (Sp-1). Next, MMP-9 is regulated at the level of the secretion of its proform. Once proMMP9 is secreted, it must be activated by removal of the prodomain (green). Finally, once activated, this activity is regulated by endogenous MMP inhibitors (TIMPs, purple), which interact with the hemopexin domain and physically block the active site.
MMP-9 in physiological and pathological roles in the periphery

While the structure of MMP-9 and its activation and regulation were fairly well worked out in the 1990s and early 2000s, new reports of functional effects of MMP-9 continue to appear. First, the majority of studies concern the role that MMP-9 plays in the remodeling and degradation of the extracellular matrix (ECM). As such, MMP-9 participates in many of the physiological events that require ECM breakdown and modification, such as cell migration, proliferation, morphogenesis, and apoptosis (Page-McCaw et al., 2007; Vandooren et al., 2013b; Vu and Werb, 2000).

Wound healing provides a good example of many of these processes. Following injury to skin, the normal path to healing involves initial hemostasis, edema formation, inflammation, and scab formation. Though MMP-9 is not constitutively expressed in epithelial cells, its expression is induced following injury. Additional MMP-9 is expressed and provided by infiltrating inflammatory macrophages (Vandooren et al., 2013b). These initial responses are followed by cell proliferation and migration, production and remodeling of the ECM, angiogenesis, and reepithelialization. This later wave of wound healing depends on MMP-9 (and other MMPs), as MMPs act not only to degrade and remodel selected ECM components, but also can liberate growth factors and bioactive fragments through targeted cleavage. The cleavage of both ECM and non-ECM molecules in order to release additional factors can ultimately influence later cellular behavior beyond simple mechanical changes in cellular architecture (Heissig et al., 2003; Mott and Werb, 2004). In fact, angiogenesis can be either stimulated or inhibited based on MMP-9’s early actions (Schultz and Wysocki, 2009).

Consistent with an overall positive role for MMPs in wound healing, it has been shown that wounds treated with MMP inhibitors heal at a delayed rate (Agren et al., 2001;
Mirastchijski et al., 2004). Further, wounds in MMP-9 null mice displayed compromised reepithelialization and reduced clearance of fibrin clots as well as exhibited abnormal matrix deposition, as evidenced by the irregular alignment of immature collagen fibers (Kyriakides et al., 2009). However, the balance is important: high concentrations of MMP-9 and high MMP-9-to–TIMP-1 ratios in wound fluid predict poor wound healing in diabetic foot ulcers (Liu et al., 2009).

Wound healing and related angiogenesis are just one example of MMP-9’s critical role in maintenance and normal physiology. MMP-9 is also important for important processes like reproduction (Dubois et al., 2000) and bone development and remodeling (Ortega et al., 2004). Further, MMPs also influence normal cellular functions when they target non-matrix proteins, including other proteinases, proteinase inhibitors, clotting factors, chemotactic molecules, latent growth factors, growth factor-binding proteins, cell surface receptors and cell-cell adhesion molecules (Tonti et al., 2009). However, when MMP-9 is dysregulated in the periphery, its actions can have a myriad of pathological consequences in diverse problems ranging from cardiovascular disease to cancer.

For example, MMPs have been implicated in muscle disease. In models of disuse-induced muscle atrophy in which either a leg is mechanically immobilized in a cast or by suspension, or by transecting the Achilles tendon, both MMP-9 and MMP-2 have been shown to be upregulated in rat gastrocnemius (Reznick et al., 2003), rat soleus (Berthon et al., 2007), as well as in wild-type mouse gastrocnemius (Liu et al., 2010c). However, though both gelatinases are upregulated to similar levels, only Mmp2 knockout mice show significantly reduced muscle atrophy when compared to wild-type littermates (Liu et al., 2010c). Another example of the role of MMP-9 comes from studies of tumor necrosis factor-related weak inducer of apoptosis
(TWEAK), which was shown to be involved in muscle atrophy (Dogra et al., 2007). Using genome wide microarray analysis of RNA samples from control and TWEAK-treated myotubes, Li et al. showed that the expression and production of MMP-9 are drastically increased in TWEAK-treated cultures and that NIK-, IKKβ-, and protein kinase 38 (p38) MAPK-dependent activation of NF-κB underlie this increased expression (Li et al., 2009). They further observed higher expression of MMP-9 in the skeletal muscle tissues of TWEAK transgenic mice and demonstrated that TWEAK-induced myopathy is significantly attenuated in Mmp9 knock-out mice.

Still, the most studied role of MMP-9 in pathological processes is its involvement in the growth and spread of cancer. In fact, MMP-9 was first identified as a result of cloning its cDNA from a tumor cell line (Huhtala et al., 1990). Two specific observations led researchers to examine the link: first, MMP-9 is overexpressed in a variety of malignant tumors, and, second, its expression and activity are often associated with tumor aggressiveness and a poor prognosis (Bauvois, 2012). Elevated levels of MMP-2 and MMP-9 are found in breast, brain, ovarian, pancreas, colorectal, bladder, prostate and lung cancers and melanoma and dysregulated MMP expression is observed in hematological malignancies such as different types of leukemia and Hodgkin's and non-Hodgkin's lymphoma (Bauvois, 2012; Vandooren et al., 2013b). In fact, MMP-9 has been implicated in shaping the microenvironment in ways that critically affect all four major hallmarks of cancer: migration, invasion, metastasis and angiogenesis (Gialeli et al., 2011). Many aspects of these processes are obviously dependent on matrix remodeling, but additional studies go beyond the proteolytic activity of MMP-9 and focus instead on its signaling properties.
First, as previously mentioned, Redondo-Muñoz and colleagues showed that the binding of proMMP-9’s hemopexin domain to the docking receptors α4β1 integrin and CD44 induces an intracellular signaling pathway that allows for the survival of CLL cells isolated from the lymphoid organ of human patients (Redondo-Muñoz et al., 2010). The authors go to show that this survival is dependent on Lyn kinase activation, STAT3 phosphorylation and upregulation of Mcl-1. High levels of proMMP-9 and Mcl-1 have also been found in CLL cells isolated from patients’ blood (Chen et al., 2010). In addition to this signaling pathway, MMP-9 multimers were shown to interact with CD44, leading to the activation of EGFR and consequently the MAPK (ERK1/2) pathway, thereby mediating cancer cell migration (Dufour et al., 2008). By the use of a small compound that fit within the loops of the barrel structures of the hemopexin domain, the formation of MMP-9 multimers could be hindered and cancer cell migration was abolished (Dufour et al., 2011). These results, and others in preclinical models, suggested that reducing MMP-9 levels either with classical inhibitors or by the use of newer compounds could have a therapeutic benefit in cancer patients. This will be discussed in more detail later.

**MMP-9 in physiological roles in the central nervous system**

MMP-9 has also been implicated in physiological and pathological roles in the central nervous system (CNS). For example, in the postnatal developing rat cerebellum, MMP-9 is expressed in migrating granule cell precursors, growing Purkinje cells, dendrites, and Bergmann glial cells (Ayoub et al., 2005; Vaillant et al., 1999) and is spatiotemporally associated with granule cell migration, axonal outgrowth, and induction of apoptosis (Vaillant et al., 2003). In the adult rodent CNS, total brain samples generally have low transcript levels for most MMPs,
but there are certain regions where high levels of constitutive MMP-9 expression are detected (Huntley, 2012).

One such region of high constitutive MMP-9 expression is the hippocampus, where MMP-9 has been shown to localize in neurons. In the hippocampus, MMP-9 plays an important role in learning and memory. When two neurons fire together, their structure and function are altered to create an enhancement of signal transduction. This process is called long-term potentiation (LTP). Hippocampal slice cultures from Mmp9 knockout mice show impaired LTP, which can be restored by the application of recombinant MMP-9 (Nagy et al., 2006). Correspondingly, Mmp9−/− mice show behavioral impairments in hippocampus-dependent associative learning (that is, pairing a footshock with a tone and quantifying the amount of time spent freezing 24 hours later), but do not have impairments in non-hippocampal learning (cued conditioning and activity suppression) (Nagy et al., 2006). In another study, transient induction of hippocampal MMP-9 was observed when animals were trained in the Morris water maze. Also, inhibition of MMP-9 activity with antisense oligonucleotides altered LTP and prevented the animals’ learning in this paradigm (Meighan et al., 2006).

Further, it has been shown that during LTP dendritic spines undergo significant remodeling in association with the long-lasting plasticity (Yuste and Bonhoeffer, 2001). In 2008, Wang et al. reported that proteolysis by MMP-9 is both necessary and sufficient to drive lasting spine enlargement and synaptic potentiation and this plasticity requires β1-integrin-dependent actin remodeling and protein synthesis (Wang et al., 2008). Specifically, they show that local application of proteolytically active MMP-9 to unstimulated dendritic spines of CA1 pyramidal neurons induces an LTP-like increase in synaptic signal strength and concurrent spine enlargement, both of which reach a maximum in the 15–20 min period after the spines are first
exposed to the enzyme. This is a specific function of MMP-9’s activity, as plasticity is not observed when spines are exposed to proMMP9 or to active MMP-2 (Huntley, 2012; Wang et al., 2008).

Also with regard to learning, it was very recently shown that MMP-9 activity in the central amygdala is required for appetitive, but not aversive, instrumental learning (Knapska et al., 2013). In that study, three kinds of appetitively motivated conditioning were impaired by either genetic deletion of Mmp9 or by blocking extracellular MMP-9 activity by nanoparticle-based release of recombinant TIMP-1 in the central amygdala. Interestingly, another recent study showed that conditional taste aversion causes a significant down-regulation in MMP-9 expression in the amygdala of rats (Panguluri et al., 2012). Further, it has been suggested that a decrease in MMP-9 levels in the hippocampus of abstinent cocaine addicts may underlie the neural deficit that makes the addict vulnerable to relapse (Mash et al., 2007).

Aside from synaptic plasticity and reward signaling, another important role for MMP-9 in the nervous system is the regulation and maintenance of Schwann cells. MMP-9 functions as a modulator of Schwann cell signaling and induces remodeling after nerve injury (Vandooren et al., 2013b). There is robust MMP-9 induction within 24 hours in both degenerating (distal) and regenerating (proximal) nerve segments of axotomized sciatic nerves (Chattopadhyay and Shubayev, 2009). Further, there is also increased Schwann cell mitosis in axotomized Mmp9−/− nerves, suggesting that MMP-9 is responsible for decreased mitosis. This inhibition of mitosis by MMP-9 was demonstrated to occur via ErbB-mediated activation of the extracellular receptor kinase (ERK) MAPK pathway in cultured Schwann cells. Finally, treatment with a broad-spectrum MMP inhibitor promotes Schwann cell proliferation (Chattopadhyay and Shubayev, 2009; Liu et al., 2010a).
Also with regard to myelination, it was shown that although initial demyelinating injury is equivalent between *Mmp9* null and wild-type mice, *Mmp9-/-* mice exhibit a significant deficit in remyelination (Larsen et al., 2003). The authors postulate that the mechanism underlying this could be the failure of MMP-9 null mice to remove injury-induced deposits of NG2 proteoglycan. Therefore, MMP-9 may have a beneficial role in remyelination.

**MMP-9 in pathological roles in the nervous system**

MMP-9 also has been linked to a number of pathological events in the nervous system including chronic neuropathic pain, stroke, blood-brain barrier (BBB) dissolution, and, of special interest here, neurodegeneration (Vandooren et al., 2013b). First, two major studies implicate MMP-9 in spinal cord-relevant pathology. Kawasaki and colleagues show a rapid and transient upregulation of MMP-9 in injured dorsal root ganglion (DRG) primary sensory neurons after sciatic nerve ligation (Kawasaki et al., 2008). They also show that MMP-9 produced in injured DRG neurons serves as one of the triggers for spinal microglial activation and neuropathic pain development and that MMP-9-induced pathophysiology involves IL-1β cleavage and microglial p38 activation. Finally, they were able to use TIMP-1 to suppress neuropathic pain.

A related study looked at the connection between opioid withdrawal and MMP-9 and found that spinal administration of exogenous MMP-9 induces morphine withdrawal-like behavioral signs and mechanical allodynia, activates NR1 and NR2 receptors, and down-regulates integrin-β1 (Liu et al., 2010b). Because a function-neutralizing antibody against integrin-β1 suppressed the MMP-9-induced phosphorylation of NR1 and NR2B and morphine withdrawal-induced MMP-9 activity is reduced by an nNOS inhibitor, the authors hypothesized that spinal MMP-9 may contribute to the development of morphine dependence primarily
through neuronal activation and interaction with NR1 and NR2B receptors via integrin-β1 and NO pathways.

Another example of MMP-9’s role in pathologies of the nervous system is that MMP-9 has been shown to be prominently involved in oxidative stress-related disruption of the blood-brain barrier (BBB) during cerebral ischemia (Gasche et al., 2001; Pustovrh et al., 2005). Mmp9−/− mice are protected from cerebral ischemia (Asahi et al., 2000) and the degradation of the critical components of the BBB and white matter after transient focal ischemia were significantly slowed in these mice compared with wild-type littermates (Asahi et al., 2001). Further, MMP-9 has also been implicated in BBB disruption in an experimental model of Huntington’s disease (Duran-Vilaregut et al., 2011). 3-NPA is a natural toxin which, when administered systemically to experimental animals, reproduces the selective neurodegeneration of striatal neurons associated with Huntington’s disease (HD). In 3-NPA-treated rats, MMP-9 was present in most of the degraded blood vessels of the injured striatum, while it was totally absent from vessels in non-injured tissue (Duran-Vilaregut et al., 2011). This is not the only link with HD; in fact, MMP-9 has been found to be upregulated in post-mortem brains of patients (Silvestroni et al., 2009) and it was also found that MMP-9 may assist in the cleavage of the huntingtin protein (Miller et al., 2010).

Other links to neurodegenerative disease have been reported (Rosenberg, 2009). Connections have been made to AD, though it remains unclear if MMP-9 is harmful or helpful in this situation. First, MMP-9 was shown to be upregulated in brain tissue of patients with AD (Backstrom et al., 1996), as well as in plasma (Lorenzl et al., 2003), but not in cerebrospinal fluid (Adair et al., 2004). These increases are thought to result from increased inflammation, and reducing inflammation has been tested unsuccessfully as a therapeutic strategy in the treatment
of AD (Rosenberg, 2009). Further, it has been demonstrated that MMP-9 plays a significant role in brain β-amyloid (Aβ) degradation and clearance. Yin et al. showed by immunohistochemistry and analysis of laser-captured mRNA that levels of MMP-9 are upregulated in activated astrocytes surrounding amyloid plaques in aged APP/PS1 transgenic mice and that MMP-9 can catabolize Aβ (Yin et al., 2006). They further showed that significant increases in the steady-state levels of Aβ are found in Mmp9+/- brains and that pharmacological inhibition of MMPs by a general MMP inhibitor increased brain interstitial fluid Aβ levels and half-life of elimination in APPsw mice. Not only can MMP-9 degrade soluble Aβ, it can also digest tightly aggregated Aβ fibrils (Yan et al., 2006). Finally, tau is a substrate of MMP-9 and the tau cleavage pattern of MMP-9 facilitates aggregation (Nübling et al., 2012).

There is also a documented link between ALS and MMP-9. First, it has been very recently reported that the C(−1562)T polymorphism in the MMP-9 gene, which leads to higher promoter activity, is significantly associated with a risk for sporadic ALS (and PD) in a Chinese population (He et al., 2013), though these results differed from an earlier study in a small Polish population that found no association between that polymorphism and risk of sporadic ALS (Zawiślak et al., 2009). Also, the total amount of both the pro and active forms of MMP-9 is higher in the serum of ALS patients than in controls (Beuche et al., 2000; Demestre et al., 2005). Further, high CSF MMP-9 levels were associated with a rapid progression of disease in human ALS patients (Fang et al., 2009). The same group found that there was also an increase in MMP-9 in homogenized skin and spinal cords of SOD1 mice only after 90 days of age, but not at early disease stages (Fang et al., 2010). Another, independent group also showed that the timing of MMP-9 induction overlapped in the spinal cord and blood of a different (low copy number) tgSOD1 mouse model, and given that 1) astrocytes have been implicated in ALS disease
progression (Di Giorgio et al., 2007; Nagai et al., 2007; Yamanaka et al., 2008) and 2) reactive astrocytes are known to secrete MMPs (Muir et al., 2002), the authors speculated that circulating MMP-9 may be derived centrally and reflect an elevated inflammatory process in the spinal cord (Soon et al., 2010),

To further explore the relationship between MMP-9 and the pathogenesis of ALS, two separate groups crossbred MMP-9 knock-out mice with mutant SOD1 mice, but got very different results (Dewil et al., 2005; Kiaei et al., 2007). First, Dewil, Robberecht and colleagues showed by zymography that astrocytes and microglia cultured from SOD1\textsuperscript{G93A} P0 pups release MMP-9. Because they also report an increase in CD11b\textsuperscript{+} and GFAP\textsuperscript{+} cells in late stage G93A spinal cords, they speculate that these are the sources of the late-stage MMP-9 induction that had been previously reported. The researchers then crossed Mmp9\textsuperscript{--/+} mutant mice with a 129 background onto a C57B1/6 background for 6 generations. These mice were then crossed with G93A mice from Jackson lab (B6SJL) for an additional 2 generations. Surprisingly, they report that their Mmp9\textsuperscript{--/+};G93A mice had significantly shorter lifespans than Mmp9\textsuperscript{+/+};G93A littermate controls (131.9 ± 2.9 days vs. 138.7 ± 3.1 days). However, they saw no influence of MMP-9 deletion on rotarod performance, despite the significant exacerbation of disease in terms of survival (Dewil et al., 2005).

In contrast, Kiaei et al. found a survival benefit of approximately 30% when they crossed Mmp9\textsuperscript{--/+} on a CD-1 background from Zena Werb’s lab (Vu et al., 1998) with G93A mice (B6SJL, from Jackson Laboratories) for 6 generations. They also report a significant preservation of motor neurons in the Mmp9\textsuperscript{--/+};G93A spinal cords at P115 (Kiaei et al., 2007). This is the earliest time-point that the authors investigated, but from these results they conclude that MMP-9 is likely contributing to the pathogenesis of ALS by 1) facilitating the cleavage of TNF-α and
related proinflammatory cytokines, or by 2) anchorage-dependent detachment of cells from the extracellular matrix. The difference between these results from the Beal group and those previously reported by the Robberecht group remain unresolved.

Though the majority of these studies have implicated glia as being the main source of MMP-9 in ALS, an early study showed that monoclonal antibodies to MMP-9 weakly labeled pyramidal neurons in the motor cortex and about 60% of motor neurons in the ventral horn of the lumbar spinal cord of both control and ALS specimens (Lim et al., 1996). Not only did this paper establish that MMP-9 is predominantly localized in the neurons, the pattern of immunoreactivity shown (cytoplasmic, extending into the dendrites and axons throughout the neuropil, but non-nuclear) as well as the proportion of positively stained motor neurons is remarkably similar to our own findings in WT and SOD1 mice (Chapter 2). Therefore, though MMP-9 has been implicated in ALS for nearly two decades, many open questions remain about whether MMP-9 is actually involved in the ALS disease pathogenesis, its site of origin and action, and the timing of its possible involvement.

The use of MMP-9 inhibitors: past, present, and future

Clearly, MMP-9 plays an important but complicated role in the organism. Its tightly regulated activity, large range of substrates, role in proteolytic processing to regulate the functions of bioactive molecules, and non-enzymatic activity make attempts to regulate specific pathologic roles of MMP-9 very challenging. The development and use of MMP inhibitors in cancer over a decade ago provides a good example of the difficulty.

As discussed earlier, MMPs were considered the prime suspects in the spread of cancer, as they could readily destroy the matrix barriers surrounding a tumor and thus permit invasion
into surrounding tissues and metastasis to distant organs. Moreover, MMPs are upregulated in virtually all human and animal tumors. Once the causal relationship between MMP overexpression and tumor progression was solidly established, many companies began developing MMP inhibitors as cancer therapeutics (Coussens et al., 2002). The first wave of MMP inhibitors were developed at a time when only 3 MMPs had been discovered (MMP-1 “collagenase”, MMP-2 “gelatinase” and MMP-3 “proteoglycanase”) and their complete set of actions were poorly understood. The compounds were further hampered by poor bioavailability (Overall and López-Otín, 2002). A next wave of inhibitors were rapidly developed that were orally available, but these too were problematic as early phase I clinical dose escalation studies revealed that prolonged treatment with these inhibitors caused musculoskeletal pain and inflammation. Although the conditions were reversible and patients were able to continue treatment after a brief drug holiday, the unexpected side effects limited the inhibitor dosages that could be administered in subsequent trials (Coussens et al., 2002).

Problems with dosage were only one of the issues with MMP inhibitors in Phase II/III trials; there were also issues with suboptimally designed endpoints as well as unexpected outcomes at this stage. The preclinical evidence suggested that MMP inhibitors would stop the growth of tumors, but not kill cells. Therefore, rather than looking for a reduction in tumor size, Phase II/III trials were designed to assess whether inhibitors like marimastat could decrease the rate of tumor markers appearance or disappearance in patients’ serum (Coussens et al., 2002; Miller et al., 2002). This was criticized as an endpoint because changes in biomarker levels in serum do not necessarily reflect tumor regression. Ultimately, marimastat failed to prolong survival when used after first-line chemotherapy for metastatic breast cancer, though the patients
did experience musculoskeletal toxicity causing the researchers to conclude that the metalloproteinases were actually being inhibited (Sparano et al., 2004).

Troublingly, two studies showed diminished survival with an MMP Inhibitor (BAY 12-9566) evaluated in pancreatic cancer and small-cell lung cancer (Moore et al., 2003; Nozaki et al., 2003). In fact, only one study has demonstrated a possible benefit for use of MMP inhibitors in cancer, showing significant improvement in progression-free survival and overall survival in a subgroup of 132 patients with unresectable or metastatic gastric or gastroesophageal adenocarcinoma. However, the study failed to meet the primary end point of improving survival in the entire group of 369 patients (Bramhall et al., 2001).

More recent studies may give clues to the failures of these trials. For example, surprisingly, general MMP inhibitors can induce the expression of MMP-9 in human fibrosarcoma cells, thereby potentially promoting rather than inhibiting tumor progression (Maquoi et al., 2002). As an additional example, following batimastat treatment, human breast carcinoma cells metastasized to the liver in nude mice and caused liver specific overexpression of MMP-2 and MMP9-9 even in tumor-free animals (Krüger et al., 2001). Therefore, the induction of organ-specific side effects may have hindered the development and clinical use of synthetic MMP inhibitors. Further, these results suggest that individual MMPs may have very different roles in cancer; that is, elevated protease levels may favor tissue-destructive processes such as those taking place in cancer, whereas specific protease deficiencies may lead to the development of many other pathological conditions (López-Otín and Matrisian, 2007). Beyond this, some have advocated a general rethinking of MMPs in cancer progression as a host response rather than an induction by tumors (Coussens et al., 2002). Generally, the field seems
to be moving away from broad spectrum inhibition and towards blocking individual MMPs under pathological conditions (López-Otín and Matrisian, 2007).

To do this for MMP-9, a few other inhibitory strategies are being pursued. For example, because of their ability to selectively bind closely related antigens, antibodies provide an excellent scaffold for creating function blocking inhibitors for individual MMPs. REGA-3G12 was among the first inhibitory antibodies reported that selectively targets the MMP-9 active site (Martens et al., 2007). However, the most recent breakthrough in MMP-specific monoclonal antibody technology was the development of “metallobodies” (Vandooren et al., 2013b). These are generated by immunizing mice with a small, synthetic, organic metal-ligand molecule—tris-imidazole zinc complex (Zn-tripod)—which was specifically designed to mimic structural and chemical motifs of the relatively exposed catalytic zinc-protein machinery in the active form of MMPs lacking their prodomain. After repetitive immunization with the Zn-tripod, further immunizations of mice with recombinant MMP-9 was used to select for highly selective monoclonal antibodies. Excitingly, these dual-specific antibodies were shown to be effective in an animal model of inflammatory bowel disease (Sela-Passwell et al., 2012).

Another strategy that is being used is a genetic one. As mentioned earlier, many experiments have demonstrated that the selective, early inhibition of MMP-9 significantly reduces the degree of brain infarction, clearly demonstrating the importance of this enzyme in contributing to focal ischemic brain injury (Yong et al., 2001). Direct delivery of siRNA against MMP-9 into the lateral ventricle of a rat brain in a middle cerebral artery occlusion model was shown to be as effective as inhibition, since disruption of BBB, brain edema, and the brain infarction volume after cerebral ischemia were significantly reduced by MMP-9 siRNA treatment (Hu et al., 2009). As another example, using siRNA oligonucleotides to knock down
the MMP-9 gene in the B16 cell line significantly inhibited tumor cell migration and invasion in vitro and caused tumor regression in vivo (Tang et al., 2013). Therefore, MMP-9 silencing also has therapeutic potential as a specific inhibitory strategy.

**Conclusions and open questions**

Given that 1) MMP-9 was identified in a previous screen as being a potential disease susceptibility factor of lumbar motor neurons in ALS and 2) it is a well-studied enzyme that has been previously linked to the function and dysfunction of the central nervous system, I wanted to ask: what can the biology of MMP-9 tell us about selective neurodegeneration? Specifically, where and when is MMP-9 expressed in wild-type as well as in mutant SOD1 mice? Further, is MMP-9 labeling a vulnerable subpopulation and, if so, can it serve as a biomarker for that susceptibility? Also, of great importance, does MMP-9 actively play a role in the disease process and if it does, by what mode of action? And finally, can we generalize the research strategy used with MMP-9 to other potential susceptibility targets?
Chapter 2: MMP-9 is selectively expressed in vulnerable motor neurons

Introduction

As discussed in the preceding chapter, there are at least two aspects of differential motor neuron vulnerability in ALS: a pool-specific resistance typified by oculomotor and Onuf’s nuclei, and a subtype-specific vulnerability of FF as compared to S motor units. Using microarray profiling of isolated wild-type motor neurons to identify genes whose expression was characteristic of both oculomotor and Onuf’s nuclei but not of vulnerable lumbar spinal neurons, or vice versa, a putative vulnerability gene, MMP-9, was identified.

MMP-9 has a restricted pattern of expression that changes from development to maturity. In the periphery, MMP-9 is frequently expressed at sites of active tissue remodeling and neovascularization. It is highly expressed during embryonic development by trophoblast cells at the implantation site and by osteoclasts. Later, it is expressed mainly by inflammatory cells and in pathological processes such as inflammatory arthritis, tumor invasion, and skin blistering diseases (Vu and Werb, 2000). In the central nervous system, MMP-9 generally has very low basal levels of expression. However, in the normal adult brain, MMP-9 mRNA has been detected in the hippocampus, cerebellum and cortex. In these areas, MMP-9 is predominantly expressed in neurons and, at the protein level, it has been found mainly in cell bodies and dendrites (Dzwonek et al., 2004). Further, a more limited level of MMP-9 expression has also been observed in glia, in particular in astrocytes and microglia, both in vivo and in vitro (Dewil et al., 2005; Kiaei et al., 2007; Oh et al., 1999; Rosenberg, 2009; Uhm et al., 1998; Vaillant et al., 1999).
It is now also well documented that excessive or inappropriate expression of MMP-9 can contribute to pathologic processes, and alterations in the expression and activity of MMPs are correlated with the development of several brain diseases (Yong et al., 2001). Increased levels of MMPs have been found in degenerative disorders such as multiple sclerosis, AD, PD and ALS diseases (Rosenberg, 2009). For most of these diseases, though it seems apparent that MMP-9 is neuronal in origin, it is not clear whether it is expressed by the neurons prone to death or those attempting to recover from the damage (Dzwonek et al., 2004). For ALS in particular, it is also not clear whether the reported increases in MMP-9 at late disease stages come from glia or from vulnerable motor neurons, though these studies strongly suggest that circulating microglia may be the source (Fang et al., 2010; Kiaei et al., 2007). However, the results of the Henderson lab oculomotor/Onuf’s nuclei microarray suggest that MMP-9 is expressed by vulnerable motor neurons themselves as early as P7 (Figure 1.5). Therefore, there are unanswered questions about the timing and localization of MMP-9 expression with regard to ALS.

Furthermore, though the microarray result suggests that MMP-9 is a potential marker for vulnerable motor neurons because it is highly expressed in L5 motor neurons but absent from resistant oculomotor and Onuf’s nuclei, how this relates to the second layer of selective vulnerability (that is, of motor neuron subtypes) is an open question. Though oculomotor and Onuf’s motor neurons have some characteristics in common with type S motor neurons (for example, small soma size, low innervation ratios, and higher levels of active duty), whether their shared molecular expression profiles extend to this resistant motor neuron subtype is not yet known. Therefore, in this chapter, we investigate the time course and specificity of MMP-9 expression in the WT mice and then relate these results to mutant SOD1 mice. In particular, we
show that MMP-9 is expressed in fast motor neurons from P5 onward. Further, we show that MMP-9 is a prospective marker for motor neurons lost at end-stage in ALS model mice.

**Results**

**MMP-9 is selectively expressed by vulnerable motor neurons**

The results of the microarray showed that the levels of *Mmp9* mRNA were 16-fold lower in oculomotor than L5 motor neurons, and 11-fold lower in DL at P7. Though this was confirmed by *in situ* hybridization (ISH) at P7 (Figure 1.5), given that ALS is an adult-onset disease, we wanted to confirm that *Mmp9* was still expressed in vulnerable motor neurons into adulthood. We performed ISH on P57 wild-type mouse cryosections and confirmed that the pattern of expression remains into adulthood; that is, *Mmp9* is highly expressed in vulnerable cranial motor pools like the trigeminal nucleus (Figure 2.1B) and the vulnerable spinal motor neurons at L5 (Figure 2.1C) and RDL in L6 (Figure 2.1D), but is absent from the resistant oculomotor pool (Figure 2.1A) and the DL nucleus at L6 (Figure 2.1D). At the protein level, using an MMP-9 antibody that gave no signal in *Mmp9* knockout mice, MMP-9 was not detected in any of the three cranial nuclei (III, IV and VI) (Figure 2.1E, and not shown) that innervate extraocular muscles, nor in the ventrolateral region of the DL nucleus in the L6 spinal cord (Figure 2.1E). In contrast, motor neurons expressing high levels of MMP-9 were abundant in trigeminal and facial nuclei as well as in L5-L6 spinal motor neurons (Figures 2.1F-G, and not shown). Therefore, MMP-9 is absent from ALS-resistant populations in midbrain and spinal cord but present in many other motor neurons. Further, in the spinal cord, strong MMP-9 motor
neuron expression is first detected only postnatally at P5 (Figure 2.2B), but subsequently remains expressed at high levels until the latest time point studied, P165 (Figures 2.2C to 2.2E).

Because MMP-9 is one of a family of MMPs, we wanted to check for the presence of other family members in motor neurons, especially the closely related gelatinase MMP-2. First, no other secreted MMPs were present as indicated by the original microarray (Fig 2.3A), and, among the probes for 20 transcripts encoding MMPs represented on the array, only Mmp9 was differentially expressed among disease-resistant and vulnerable populations. Furthermore, besides Mmp9, only four other MMPs appeared to have high expression values in motor neurons: Mmp15, Mmp16, Mmp17, and Mmp24. These are all members of the same sub-family of non-secreted, membrane-type MT-MMPs (Yong, 2005). Also, as a second check beyond the microarray data, MMP-2 was confirmed to not be expressed in the adult mouse lumbar spinal cord at the level of L5 (where MMP-9 is strongly expressed in approximately 60% of motor neurons), nor in the DL nucleus (where MMP-9 levels are very low) (Fig 2.3 C, G). Somewhat surprisingly, levels of TIMP2, which regulates MMP-2, are very high (Fig 2.3D, H), whereas TIMP3, which can inhibit both MMP-2 and MMP-9, is only moderately expressed (Figure 2.3 E, I).

Given the high expression of MMP-9 in motor neurons, we wanted to know if it also was expressed in other motor relevant cell types in postnatal mice. First, IHC of a whole coronal section of the lumbar region of spinal cord shows that MMP-9 protein is expressed strongly by motor neurons in the ventral horn, but by few other neurons in the spinal cord and only very weak dorsal horn staining is observed (Figure 2.4A). This image is from a presymptomatic (P25) SOD1\(^{G93A}\) animal, which we have found to have the same expression pattern as wild-type littermates prior to disease onset (compare ventral horns shown in Figure 2.4B and C). Mutant
SOD1 does not therefore itself trigger misexpression of the enzyme. Further, we confirmed the absence of MMP-9 in proprioceptive dorsal root ganglia from WT and SOD1\textsuperscript{G93A} mice, identified by labeling with neurofilament (Figures 2.4D and E). Mmp9 RNA is also absent from muscle sections of these mice (Figures 2.4F and G). Finally, given that MMP-9 is a secreted enzyme, we checked the axons of a SOD1\textsuperscript{G93A} mouse at P40 for MMP-9 and found that it was expressed there (Figure 2.4H), though we did not see MMP-9 at the NMJ. Therefore, MMP-9 is specifically expressed in postnatal, disease-susceptible motor neurons.

**MMP-9 is selectively expressed by fast motor neurons**

Even within motor neuron populations that expressed MMP-9, strongly labeled cells were intermingled with MMP-9-negative neurons (Figures 2.4B, 2.5). Additionally, the MMP-9 staining pattern in L5 motor neurons was not completely uniform; some motor neurons clearly exhibited high intensities of MMP-9 staining (termed “MMP9\textsuperscript{hi}”), while others exhibited only moderate levels of staining (termed “MMP9\textsuperscript{lo}”). Still others were completely devoid of MMP-9 staining, and were thus termed MMP-9\textsuperscript{neg} (Figure 2.5A, B). To further explore this heterogeneity, we quantified the relative proportions of MMP-9\textsuperscript{hi}, MMP-9\textsuperscript{lo}, and MMP-9\textsuperscript{neg} motor neurons at L5 and in the DL and RDL nuclei at L6. To confirm our observation that the MMP-9 staining pattern fell into the 3 categories that we assigned, we first used Image J software (Windows version; National Institutes of Health) to quantify the intensity of MMP-9 staining in every L5 motor neuron from one animal. We determined that the spread of absolute intensity values fitted best to a 3\textsuperscript{rd} order polynomial function. This suggested that these values can be separated into three distinct bins with thresholds at the two points of the function inflection corresponding, from lowest to highest, to MMP-9\textsuperscript{neg}, MMP-9\textsuperscript{lo}, and MMP-9\textsuperscript{hi} distinctions we obtained by visual inspection (Figure 2.5D). We then performed manual counts using the same set of images.
Reassuringly, we found that the total counts of motor neurons with MMP-9 intensities within each bin were very similar whether measured by the software image analysis or by manual counts (Figure 2.5E). We therefore went on to count manually the relative abundance of each MMP-9 staining category in 3-4 WT animals. Of all ChAT positive motor neurons at L5, 42.6 ± 1.6% were MMP-9\(^{hi}\), 22.1 ± 1.1% were MMP-9\(^{lo}\) and the remaining 35.1 ± 1.2% did not express MMP-9 (mean ± s.e.m, n=4). In L6, the overall distribution of MMP-9 intensities in the RDL nucleus were similar to those at L5 (MMP9\(^{hi}\): 24.6 ± 4.8%; MMP-9\(^{lo}\): 30.0 ± 1.2%; and MMP-9\(^{neg}\): 45.4 ± 5.3%). In contrast, the resistant DL nucleus showed significantly less MMP-9 expression than L5 or RDL motor neurons: 70.0 ± 3.6% of DL motor neurons were MMP-9\(^{neg}\) while only 19.5 ± 2.6% were MMP-9\(^{hi}\), and 10.5 ± 1.8% MMP-9\(^{lo}\) (mean± s.e.m, n=3) (Figure 2.5C). Given the very low variability between animals, we hypothesized that MMP-9 labels defined subpopulations of lumbar motor neurons. We therefore sought to determine whether the ALS-vulnerable population labeled by MMP-9 might correspond to a previously identified functional subtype.

Given that MMP-9 was present in susceptible pools, and that FF/FR motor neurons have been shown to be more disease-vulnerable than S motor neurons (Pun et al., 2006), we asked whether the MMP-9-expressing neurons might be fast alpha-motor neurons. To test whether MMP-9 is expressed by a particular class of motor neurons (alpha or gamma), we performed triple immunolabeling for MMP-9, ChAT and NeuN to distinguish between ChAT\(^{+}\) NeuN\(^{+}\) putative alpha motor neurons and ChAT\(^{+}\) NeuN\(^{-}\) gamma motor neurons (Friese et al., 2009; Shneider et al., 2009). First, we found that 17.5±1.5% of all ChAT\(^{+}\) motor neurons at L5 were NeuN\(^{-}\) (mean± s.e.m, n=3) and, in agreement with earlier reports, these putative gamma MNs tended to be the smallest ChAT\(^{+}\) cells (Figure 2.6A, arrow). Further, every ChAT\(^{+}\)NeuN\(^{-}\) motor
neuron lacked MMP-9 immunoreactivity (Figure 2.6B-C). This suggests that gamma motor neurons, like the disease-resistant extraocular and pelvic motor pools, do not express MMP-9.

Of the remaining alpha motor neurons at this level, not all were MMP-9 positive, meaning that MMP-9 is not a general alpha motor neuron marker. To identify if MMP-9 expression marked a given alpha motor neuron subtype, we undertook 3 additional analyses. First, a characteristic of FF/FR motor neurons is that they are larger than S motor neurons (Cullheim et al., 1987; Kanning et al., 2010). We limited our analysis to putative α-motor neurons co-stained for NeuN in a random sampling of L5 of WT P40 (n=3 animals, approximately 75 cells from each, Figure 2.6D). All 3 populations of motor neurons (hi, lo, and neg) had normally distributed cell soma sizes (Figure 2.6 E). The MMP-9 hi population was the largest (the mean ± SEM was 1193 ± 31 µm² versus 946 ± 23 µm² for MMP-9 lo and 725 ± 27 µm² for the MMP-9 neg population). Though FF, FR, and S motor neuron subtypes exist along a spectrum, it was tempting to propose from these data that MMP-9 hi cells are FF, MMP-9 lo are FR, and MMP-9 neg are S. We therefore conducted two additional sets of experiments to further correlate MMP-9 intensity and motor neuron subtype.

We identified motor neurons of the slow soleus (Sol) and fast tibialis anterior (TA) pools by injection of retrograde tracer into each muscle in adult mice (Figures 2.7A-F). The absolute values for MMP-9+ (MMP-9 hi and MMP-9 lo combined) and MMP-9 neg abundance closely matched the percentage of fast and slow fiber types, respectively, in Sol (53% MMP-9+; 54% FF+FR) and TA (90% MMP-9+; 93% FF+FR) muscles (Hegedus et al., 2007). It was also reported that the most lateral subcompartment of the lateral gastrocnemius (GC-L1) is innervated exclusively by FF motor neurons (Pun et al., 2006). We hypothesized that if we injected CTB-488 into GC-L1, the corresponding motor pool would be made up entirely of MMP-9 hi motor
neurons. Though we confirmed that the majority of the labeled pool was MMP-9⁺, there were MMP-9⁻⁰ and MMP-9⁻ motor neurons as well (Figure 2.7G). One potential explanation for this comes from the fact that, in contrast with the situation with the TA and Sol muscles for which we were able to confirm that tracer remained in the targeted muscle, all 3 animals that received GC-L1 injections appeared to have some level of tracer leakage to adjacent muscle subcompartments.

As a final indicator of fast vs. slow, we analyzed how MMP-9-labeled cells compared to those labeled with putative markers of fast type motor neurons (Enjin et al., 2010) and type S motor neurons (Chakkalakal et al., 2010) (Figure 2.8). Because we found that the commercially available antibody to CHODL resulted in non-specific staining, we took advantage of Chodl⁺/⁻ mice with the LacZ gene expressed in Chodl-positive cells. When we stained for β-galactosidase and MMP-9 in cryosections of lumbar spinal cord, we found that a subset of motor neurons (76%) were positive for β-galactosidase, and thus putatively CHODL, and that almost all MMP-9 positive motor neurons were also CHODL positive (Figure 2.8A-B). In fact, of all MMP-9⁺ motor neurons, 96% also expressed the CHODL-lacZ reporter, and only 29% of lacZ⁺ cells lacked MMP-9. This could potentially indicate that MMP-9 labels a subset of fast motor neurons, but it is equally possible that the Chodl reporter is also expressed in some other motor neurons. Since the Chodl⁺/⁻ mice have not yet been well characterized, we also looked in WT cord to see how the MMP-9⁺ population of motor neurons compared to Chodl⁺ or Sv2a⁺ cells by ISH. We analyzed directly adjacent cryosections of L3 spinal cord (each 12 µm thick) for expression of Chodl, Mmp9, and Sv2a, and found that Chodl and Mmp9 appeared to be expressed in similar populations, but Sv2a did not (Figure2.8C-E). We were not successful in performing double labeling for MMP-9 IR and Chodl ISH on the same section but in the future this, or
double *in situ* hybridization, should further confirm our findings. Overall our data show that MMP-9 is selectively expressed in fast α-motor neurons.

**MMP-9 as the first prospective marker for vulnerable motor neurons in mouse models of ALS**

Most of the experiments described thus far had been performed in WT animals and suggested that MMP-9 is a prospective marker for vulnerable motor neurons in ALS. To test this in an animal model of ALS, we decided to look at end-stage SOD1<sup>G93A</sup> mice (end-stage is defined as when the animal can no longer right itself within 30 seconds after being turned on its back, approximately P157 in our colony). For a series of motor neuron subpopulations we compared the percentage of motor neurons lost at end-stage in SOD1<sup>G93A</sup> mice (Ferrucci et al., 2010) to the fraction that expressed MMP-9 in wild-type mice (Figure 2.9A). Absolute values in the two datasets showed a remarkably strong positive correlation ($y = 1.07x + 3.99$, $R^2 > 0.96$), suggesting that motor neurons that express MMP-9 are those subsequently lost in ALS. Further, when we compared the fraction of neurons in a given motor pool that express MMP-9, identified by retrograde labeling (Figure 2.7), and the innervation of the corresponding muscle at end-stage in SOD1<sup>G93A</sup> mice, it appears the relationship also extends to axonal dieback (Fig. 2.9B).

Therefore, in motor pools that have high baseline MMP-9 expression levels such as L5, there is a massive loss of motor neuron in ALS (Figure 2.10 B-C, E). In fact, at this level, only ~50% of motor neurons remain, and all of these were MMP-9-negative (Figures 2.10B, D, and E). This suggested that MMP-9-expressing motor neurons are selectively lost during the ALS disease process. However, at P75, prior to any significant motor neuron loss, the average number of MMP-9<sup>neg</sup> motor neurons significantly increased from $6.5 \pm 0.4$ to $9.8 \pm 0.1$ per
ventral horn (p<0.001, Student’s t test). This small but significant increase suggests that MMP-9 down-regulates prior to cell death. However, the absolute number of surviving motor neurons in L5 spinal cord at end-stage was not significantly different from the number of MMP-9-negative cells initially present (Figure 2.10E; p=0.62, t-test). This, taken together with the strong correlation between MMP-9 expression and overall vulnerability (Figure 2.9), points to MMP-9 as the first prospective marker for vulnerable motor neurons in mouse models of ALS.

Interestingly, at end-stage MMP-9 could be detected in microglia (Figure 2.10F), and this could be why other groups have reported an increase in MMP-9 levels in late-stage mice and patients (Fang et al., 2009; Kiaei et al., 2007), despite the fact that there are no longer MMP-9 positive MNs.

As a final way to correlate MMP-9 positivity and disease susceptibility in SOD1G93A mice, we decided to look at one of the earliest molecular changes that take place in vulnerable motor neurons: ER stress. We asked how the early wave of UPR detected by phosphorylation of eukaryotic initiation factor-2α (P-EIF2α) was localized relative to the MMP-9+ population of motor neurons. As expected (Saxena et al., 2009), we observed a strong increase in P-EIF2α staining between P30 and P40, from 1.2 ± 0.29 MNs per ventral horn to 12.4 ± 1.1 (mean ± s.e.m, n=5, Fig. 2.11D-F). When we quantified the overlap with MMP-9 immunoreactivity (Figure 2.11G), the number of double-labeled cells was significantly greater than expected by chance; n=5, χ²=3.96, d.f.=1, p<0.05). Therefore ER stress is likely selectively induced in MMP-9+ motor neurons, in agreement with their FF/FR identity.

**The MMP-9 expression pattern in human patients remains an open question**

The preceding data strongly suggest that MMP-9 can serve as a biomarker for vulnerable motor neurons in mice. In order to assess the relevance to human patients, we wanted to check
the constitutive expression levels of MMP-9 in human spinal cords. Because all available tissue from ALS patients comes from postmortem examination, our results in mice predict that at that time point there would have been massive cell death and the MMP-9 motor neurons would already be lost (Figure 2.10). Therefore, we decided to analyze MMP-9 expression in a non-ALS, control patient. The NY Brain Bank provided tissue samples from 2 sources: the midbrain and lumbar and sacral spinal cord from a 78 year-old male patient who had been diagnosed with AD and the lumbar spinal cord of a 60 year-old male patient who died of non-neurological complications. Unfortunately, the tissues from the first patient gave no immunofluorescence signal in any condition with any antibody tested. We hypothesize that the fixation of these tissues (fresh frozen) was suboptimal for immunohistochemistry.

The second spinal cord sample had been formalin fixed and paraffin embedded, and was therefore considered a more reliable sample for a second round of immunostaining. H&E staining identified large chromatolytic motor neurons (Figure 2.12A). However, the cytoplasm of these motor neurons did not stain for MMP-9 with the same pattern as would be predicted based on the pattern in the mouse spinal cord (compare 2.4A and 2.12B). Interestingly, when compared to the secondary antibody-only control on an adjacent paraffin section (Figure 2.12C), there does appear to be a generally higher amount of brown signal, possibly suggesting staining of the processes. It was possible that the antibody was faulty, so we confirmed its specificity using a section of spleen from a different patient (generously provided by Dr. Kara Spiller, lab of Gordana Vunjak-Novakovic, Figure 2.12D). Given that the antibody worked in the positive control, we can imagine a few scenarios that account for the lack of motor neuron staining. First, it is possible that MMP-9 is not expressed by human motor neurons, despite an earlier report to the contrary (Lim et al., 1996). It could also be that we did not see MMP-9 in this patient.
because it is not expressed constitutively, but only on demand as in most other cell types (Nagase et al., 2006). Another possibility, is that there is more variability in MMP-9 levels in humans, but with only an n=1 we cannot detect it. Finally, MMP-9 expression may down-regulate with age. Follow-up studies in more human tissues are needed.

Discussion

In this chapter, we show that MMP-9 is predominantly expressed by motor neurons and weakly by a small population of dorsal interneurons in the mouse spinal cord. There are 3 types of motor neurons (alpha, beta, and gamma), which are classified based on which type of muscle fiber they innervate. Based on size and co-labeling with NeuN, we have shown that MMP-9 is only expressed by alpha motor neurons, those that innervate extrafusal muscle fibers and directly initiate movement. Alpha motor neurons constitute the largest population of motor neurons in the spinal cord and can be further subdivided based on the functional characteristics of their target muscles: fast-twitch fatigable (FF), fast-twitch fatigue-resistant (FR), and slow-twitch fatigue-resistant (S) (reviewed in Chapter 1). These three MN subtypes differ with respect to soma size, morphology, and central connectivity, and importantly, in their vulnerability to disease (Kanning et al., 2010). Because of this differential vulnerability, and to better understand basic motor neuron biology, it is useful to have molecular markers that could further distinguish these MN subtypes from each other.

Numerous groups have attempted to find such markers in recent years. For example, Chakkalakal et al. provided evidence that the synaptic vesicle protein Sv2a is selectively expressed by slow MNs and specifically accumulates in motor axon terminals on slow muscle
fibers (Chakkalakal et al., 2010). In the same year, Enjin et al. found that slow motor neurons were also labeled by Err2 (Enjin et al., 2010). That paper also showed that chondrolectin (Chodl) had a complementary pattern of expression to Err2. Moreover, ISH for Chodl labeled mostly large cells and patch-clamp recordings from Chodl-positive motor neurons showed that these cells have electrophysiological properties consistent with fast motor neurons. Excitingly, based on the results presented in this chapter, we now feel it is appropriate to add MMP-9 to the set as a fast motor neuron marker.

We demonstrated the specific presence of MMP-9 in this subpopulation using 3 different approaches: (1) co-labeling and/or labeling adjacent sections with a known fast and a known slow marker, (2) soma size comparisons, and (3) examining the MMP-9 staining levels in motor pools with known proportions of FF, FR, or S motor neurons (approximated by analyzing the fiber type composition of the target muscles). Comparison of the MMP-9-positive motor neuron population with those expressing Chodl and Sv2a suggests that MMP-9 is a fast but not slow marker. Moreover, the other 2 experiments imply that the intensity of MMP-9 immunopositivity could further subdivide the fast category.

Given that the motor neuron subtypes are known to differ in size with FF being the largest, S being the smallest, and FR MNs being intermediate (Cullheim et al., 1987), it is tempting to hypothesize based on the distribution of soma areas of α-MNs (ChAT+/NeuN+) as a function of MMP-9 immunoreactivity that MMP-9^{hi} cells are the FF subtype, MMP-9^{lo} label the FR subtype, and the S subtype is MMP-9^{neg}. As another check, we ideally wanted to quantify MMP-9 levels in motor pools with relatively uniform subtype compositions, for example the GC-L1 been reported to be composed of nearly 100% FF motor neurons (Pun et al., 2006). The prediction was that this motor pool would be comprised of only MMP-9^{hi} cells. However,
although the majority of CTB positive cells were MMP-9 positive after injection into the lateral subcompartment, there were some MMP-9$^{\text{lo}}$ and MMP-9$^{\text{neg}}$ cells labeled. Unfortunately, this experiment was hampered by difficulty in the specificity of the injection, and we could detect fluorescence in neighboring muscle fibers outside of GC-L1.

The injections in the SOL and TA, on the other hand, were determined to be much more specific to just those muscles, and the proportions of MMP-9$^{\text{hi}}$, MMP-9$^{\text{lo}}$, and MMP-9$^{\text{neg}}$ motor neurons matched very closely with the fibers types reported in those pools by an independent group (Figure 2.7G and H). Specifically, we found that on average highly vulnerable TA motor neurons contained more MMP-9$^{\text{hi}}$ than MMP-9$^{\text{lo}}$ and virtually no MMP-9$^{\text{neg}}$ motor neurons, while more resistant Sol motor neurons contained only a small percentage of MMP-9$^{\text{hi}}$ motor neurons, and were mostly composed of MMP-9$^{\text{lo}}$ and MMP-9$^{\text{neg}}$ motor neurons. Therefore, taken together our results raise the possibility that within generally vulnerable lumbar motor pools, MMP-9$^{\text{hi}}$ motor neurons are those that undergo early degeneration, MMP-9$^{\text{lo}}$ succumb later, and MMP-9$^{\text{neg}}$ motor neurons, likely corresponding to a mixture of type S and gamma motor neurons, are those that survive until end-stage.

Though both are reliable fast motor pool identifiers, MMP-9 has several advantages over the other candidate fast marker Chodl as a marker for selectively vulnerable cells. First, Chodl is hard to detect by immunostaining whereas MMP-9 has at least 3 different commercially available antibodies that give strong, consistent signals and do not stain MMP-9 knock-out tissue. Further, specific motor neuron MMP-9 expression begins only around P5 (Figure 2.2), an age just before the first changes in mSOD1 mice can be detected suggesting that MMP-9 expression could be influencing motor neuron vulnerability (Kanning et al., 2010). On the other hand, Chodl mRNA is expressed both in motor neurons and at the base of the developing limb at E10.5.
and E11.5, the developmental stages when motor axons reach this region and make a number of
decisions, suggesting a possible function of chondrolectin in guiding axons at this choice point
(Enjin et al., 2010). Further, it has been recently shown that Chodl is an in vivo genetic modifier
of the SMA phenotype, as results from smn-depleted zebrafish suggest the possibility that
upregulation of chondrolectin levels could protect motor neurons in SMA (Sleigh et al., 2013).
For these reasons, we argue that MMP-9 is a better marker of vulnerable motor neurons.

The microarray profiling that originally identified MMP-9 was done following the
hypothesis that there could be a set of cell-intrinsic properties that render certain motor neurons
more susceptible to damage than others. We initially hypothesized that MMP-9 could be a
putative susceptibility factor in motor neuron disease based on its absence from oculomotor and
Onuf’s nuclei, and found it striking that its absence also extended to resistant S motor neurons.
Among a group of neurons that are diverse by their ontogeny and function, it may be significant
that all three resistant populations share characteristics other than lack of MMP-9 expression.
The extraocular-innervating and the pelvic sphincter-innervating populations, like S motor
neurons, have small cell bodies and axonal arbors and slower-conducting axons, and all fire at
constant rates for extended periods of time (Kanning et al., 2010). Despite these shared
functional properties and shared lack of MMP-9, they do not necessarily fully share molecular
profiles. For example, CGRP has been shown to be expressed by larger diameter, more
susceptible motor neurons, with 90% of CGRP-expressing motor neurons being lost in mutant
SOD1 mice compared to the 50% degeneration of ChAT+ motor neurons (Piehl et al 1993; Vlug
et al 2005). However, both extraocular and pelvic motor pools express high levels of the Calca
transcript encoding CGRP (Allen Brain Atlas). This suggests that CGRP may be a marker for
fast motor neurons, but shows that its absence does not reliably predict resistance. Therefore,
MMP-9 may be unique in its absence from all small, extended-firing motor neurons and future studies to determine whether the presence or absence of MMP-9 affects functional properties such as motor neuron size and/or electrical properties will be of great interest.

Having demonstrated that MMP-9 was selectively expressed in WT mice in motor neurons known to die in disease, we next extended the MMP-9 expression analysis to the best model of familial ALS available: mutant SOD1 mice. We found a remarkable degree of positive correlation between the fraction of wild-type motor neurons expressing MMP-9 in a given spinal or brainstem region and the degree of susceptibility of that population to degeneration in the SOD1\textsuperscript{G93A} mice, suggesting that MMP-9 could be a prospective marker for future cell loss. Correspondingly, at end-stage in SOD1\textsuperscript{G93A} mice, the majority of the remaining cells (~50%) were negative for MMP-9. Finally, it has been elegantly shown that in mutant SOD1 mice, large FF motor neurons generate an unfolded protein response before fatigue-resistant or slow motor neurons do and it has been hypothesized that this sequence of events may reflect a difference in the stress-coping capacity of these different types of neurons (Saxena et al., 2009; Saxena and Caroni, 2011). Consistent with their identity as FF motor neurons, we show that upregulation of phosphorylated EIF2α occurs selectively in MMP-9-positive motor neurons at P40. The relationship between ER stress and MMP-9 will be further addressed in Chapter 4.

Taken together, these data distinguish MMP-9 as the most reliable biomarker for motor neuron susceptibility to ALS to date. Given selective expression of MMP-9 in motor neurons in the spinal cord, and its exclusion from oculomotor and Onuf’s nuclei, and from the slow, ALS-resistant subtype in spinal motor pools, MMP-9 provides a unifying hypothesis for class-, pool- and subtype-specific aspects of selective motor neuron resistance to ALS. This striking expression pattern of MMP-9 suggests that this protein itself could be playing a role in the
degeneration of vulnerable motor neurons in mutant SOD1 mice. This hypothesis will be addressed in a series of experiments in the following chapter.
Figures

Figure 2.1

<table>
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- **A**: Image of oculomotor area.
- **B**: Image of trigeminal motor area.
- **C**: Image of L5 area.
- **D**: Image of L6 area.
- **E**: Detailed view of oculomotor area.
- **F**: Detailed view of trigeminal motor area.
- **G**: Detailed view of L5 area.
- **H**: Detailed view of L6 area.
Figure 2.1 MMP-9 is expressed in subsets of cranial and spinal motor neurons but is absent from oculomotor and DL nuclei.

A-D.ISH for Mmp9 on cryosections of wild-type brainstem and spinal cord at P57 showing similar results to those at P7 (Figure 1.5C). Mmp9 is expressed at different levels in subsets of trigeminal motor nucleus (B) and L5 (C) motor neurons. Oculomotor (A) and DL nucleus motor neurons (D) do not express Mmp9.

E-H. Immunostaining for MMP-9 (green) on P30 spinal cord of ChAT-Cre; Rosa-TdTomato (MN, red) reporter mice shows many motor neurons expressing high levels of MMP-9 in the vulnerable trigeminal nucleus (F), L5 spinal cord (G) and RDL in L6 (H). In contrast, resistant oculomotor (E) and DL (H) nuclei show no or very little MMP-9 expression.

Collaboration with A. Kaplan.
Figure 2.2
Figure 2.2. Time course of MMP-9 expression in the non-transgenic mouse lumbar spinal cord

A-D. ISH for Mmp9 on cryosections of wild-type lumbar spinal cords at various indicated ages reveals that MMP-9 is first detected postnatally at P5 (B), and subsequently remains expressed at high levels until the latest time point studied, P165 (E).
Figure 2.3

A

![Graph showing relative expression of different MMPs across different conditions.]

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B

Chat

C

Mmp2

D

Timp2

E

Timp3

F

L4-L5

G

RDL

H

RDL

I

RDL

J

RDL

K

RDL

L

RDL
Figure 2.3 Expression of other MMP family members and their endogenous inhibitors in motor neurons

A. MMP-9 is the only secreted MMP expressed at high levels in motor neurons at P7. Raw expression values for indicated probes on microarrays for DL, III, and L5 samples were divided by the raw expression value of Mmp9 in L5 motor neurons. Transcripts were arranged into known subgroups based on protein domains and substrate specificity including: gelatinases (Mmp9 and Mmp2), collagenases (Mmp1a, Mmp1b, Mmp8, Mmp13), stromelysins (Mmp3, Mmp10, Mmp11), matrilysins (Mmp7) and membrane-type (MT–MMPs) (Mmp14, Mmp15, Mmp16, Mmp17, Mmp24), and other (Mmp12, Mmp19, Mmp20, Mmp23). Only members of the MT-MMP subgroup showed similar or higher expression levels as compared to Mmp9 whereas all other MMPs, including Mmp2, were nearly absent. Mmp15 and Mmp17 had >4-fold higher expression values compared to Mmp9 but were not specific to L5 motor neurons. 

Collaboration with A. Kaplan.

B, F. Adjacent cryosections were processed for Chat ISH to identify motor neurons at L5 (B) and DL and RDL nuclei at L6 (F).

C,G. Mmp2 is absent from cryosections of adult L5 and L6 spinal cord, as detected by ISH.

D,H. ISH for Timp2 on cryosections of adult, wild-type L5 (D) and L6 (H) shows that Timp2 is strongly expressed by all neurons.

E, I. ISH for Timp3 on cryosections of adult, wild-type L5 (E) and L6 (I) shows that Timp3 has generally low expression levels but appears to be expressed more strongly by subsets of RDL motor neurons.
**Figure 2.4 MMP-9 is specific for motor neurons within the neuromuscular system**

A. Representative coronal section from a P25 SOD1<sup>G93A</sup> mouse lumbar spinal cord immunostained for MMP-9. Intense MMP-9 staining is visible in motor neurons in the ventral horn, as well as some very weakly stained cells in the dorsal horn (arrows). This pattern is the same as in WT mouse lumbar spinal cord (B). Scale bar 100 μm.

B-C. L5 spinal cord of SOD1<sup>G93A</sup> (C) and age-matched wildtype control (B) at P40 immunostained for MMP-9 (green) and VACHT (red) shows that the presence of mutant SOD1 does not affect MMP-9 expression.

D-E. MMP-9 is absent from DRGs; no MMP-9 staining (red) was detected in dorsal root ganglia neurons detected by staining for neurofilament (NF, green) from a P30 WT (D) or SOD1<sup>G93A</sup> (E).

F-G. *In situ* hybridization for Mmp9 on cryosections of P50 WT (F) and P60 G93A (G) TA shows that no signal is detected in that muscle.

H. MMP-9 (green) is present in some axons of a P40 SOD1<sup>G93A</sup> lumbar level ventral root, indicating that it may be secreted.
Figure 2.5
Figure 2.5 MMP-9 is expressed at variable levels in disease-vulnerable pools of lumbar motor neurons.

A, B. Representative images showing levels of MMP-9 expression (red) categorized as being high (arrow), low (arrowhead), or negative (asterisk) at L5 in a wildtype P75 mouse. B, Motor neuron cell bodies are visualized using ChAT immunostaining (green). Scale bar, 20 μm.

C. Quantification of MMP-9 staining intensities in L5, DL and RDL motor neurons (mean ± s.e.m, n=3-4). Resistant DL nucleus motor neurons expressed significantly lower levels of MMP-9 than did vulnerable L5 or RDL. A two-way analysis of variance showed that differences between motor neuron subsets were significant [F(4, 29) = 34.1, p<0.001]. Post hoc comparisons using the Bonferroni analysis revealed that the DL nucleus contained a significantly higher proportion of MMP-9neg motor neurons than L5 (t= 9.06, p<0.01) or RDL (t= 6.00, p<0.01).

D. Scatter plot of MMP-9 signal corrected for background in a fixed area within each of 273 motor neurons in the L5 spinal cord of one animal. A 3rd order polynomial best fit trend line (red), determined by maximizing the R² value (>0.98), was added and the second derivative was taken to find two inflection points of the function. The ordinate values of the two inflection points were set to be the bin thresholds. All motor neurons with MMP-9 intensity lower than the first inflection point were considered MMP-9neg, those between the two inflection points were considered MMP-9lo, while all above were considered MMP-9hi.

E. Close correspondence between automated and manual counts. Fraction of each category in L5 calculated by automated image analysis in D (ImageJ) or by manual counting in C (Manual).
Figure 2.6

MMP-9 NeuN ChAT

A

B

C

D

E

% MMP-9 MN

NeuN+ NeuN−

MMP-9hi

MMP-9lo

MMP-9neg

% α-MN within range

soma area (µm²)

0 5 10 15 20 25 30 35

Area = 732.59 µm²
Area = 531.08 µm²
Area = 826.70 µm²
Area = 1270.36 µm²
Area = 1333.02 µm²
Area = 1271.17 µm²
Area = 615.20 µm²
Area = 1439.32 µm²
Area = 1780.93 µm²
Figure 2.6 The largest alpha motor neurons express MMP-9

A,B. Representative images of triple labeling of L5 spinal cord cryosection with MMP-9 (red), ChAT (blue), and NeuN (green). Putative small-diameter ChAT⁺ NeuN⁻ gamma motor neurons are denoted by arrows. Note the absence of MMP-9 staining in these motor neurons (B, arrows).

C. Quantification of MMP-9 staining intensity in putative alpha and gamma motor neurons. Of the ChAT⁺NeuN⁺ alpha motor neurons, 53.3 ± 1.2% were MMP-9hi, 25.6 ± 0.5% were MMP-9lo, while 21.1 ± 0.9% were MMP-9neg. In contrast, none of the ChAT⁺NeuN⁻ gamma motor neurons were found to express MMP-9. Values are means ± s.e.m of counts performed in three animals.

D. Example of soma size quantification from an image of L5 spinal cord cryosection labeled with MMP-9 (red), ChAT (blue), and NeuN (green).

E. Distribution of soma areas of α-MNs (ChAT⁺/NeuN⁺) as a function of MMP-9 immunoreactivity in lumbar spinal cord from 3 WT animals at P40. MMP-9 staining intensity was determined as in Figure 2.5. Motor neurons expressing MMP-9 at the highest level (black bars) are the largest, consistent with a fast identity. MMP-9-negative MNs were the smallest (white bars) and are likely to be slow MNs.
Figure 2.7

(A) NeuN CTB MMP-9

(B) TA

(C) Sol

(D) NeuN CTB MMP-9

(E) TA

(F) Sol

(G) % MNs expressing MMP-9

(H) % fiber type of target muscle

Pun et al., 2006

Hegedus et al., 2007
Figure 2.7 The slow soleus motor pool contains fewer MMP-9+ motor neurons as compared to the fast tibialis anterior and lateral gastrocnemius motor pools

A-F. MMP-9 immunostaining (red) on alpha motor neurons labeled with NeuN (blue) of the TA and Sol motor pools of adult WT mice identified by retrograde labeling with CTB488 (green). Many motor neurons of the fast TA pool are intensely MMP-9-positive, whereas Sol motor neurons tend to be negative or express low levels of MMP-9.

G. Quantification of MMP-9 staining in the GC, TA and Sol motor pools (mean ± s.e.m., n=3) shows a larger fraction of MMP-9-negative motor neurons in the slow Sol pool as compared to the faster TA and lateral subcompartment of GC.

H. Percentages of type I (slow, light grey) and type IIa (FR, dark grey) and II b (FF, black) fibers in GC-L1, TA and Sol muscles replotted from Pun et al., 2006 and Hegedus et al., 2007 as an indicator of subtype composition of the corresponding motor pools. Values for fast fibers are very close to those for MMP-9 for TA and Sol, but not GC-L1, likely reflecting leakage of retrograde tracer into adjacent muscle subcompartments.

Collaboration with A. Kaplan.
Figure 2.8
Figure 2.8  MMP-9 labels a similar subpopulation of motor neurons as the previously identified fast molecular marker CHODL

A, B. Immunostaining for VACHT (green), MMP-9 (red), and LacZ (blue) on adult spinal cord of CHODL: lacZ mouse. A subset of VACHT+ MNs are LacZ-positive, and a subset of those (all but 29%) are also MMP-9-positive.

C-E. Adjacent cryosections of lumbar WT P40 spinal cord were processed by ISH for Chodl (C) to identify fast motor neurons and Sv2a (E) to identify slow motor neurons. The pattern of MMP-9 expression (D) looks much more similar to Chodl than to Sv2a.
Figure 2.9
Figure 2.9 MMP-9 expression in wild-type motor neurons correlates tightly with levels of degeneration in mutant SOD1^{G93A} mice at end-stage.

A. Tight correlation between MMP-9 expression in WT and ALS vulnerability. For a given motor neuron population, percentages of motor neurons expressing MMP-9 (both high and low levels) were plotted on the y-axis, and percentages lost in SOD1^{G93A} mice at end-stage as counted by us or presented by Ferucci et al. plotted on the x-axis (2010). Data were fitted with a linear regression (y = 1.07x + 3.99, R^2 >0.96). Values are mean ± s.e.m. (n=3). Collaboration with A. Kaplan.

B. Quantification of MMP-9-negative motor neurons (black bars) retrogradely labeled from different muscles (Sol, EOM, GC-L1, and TA; indicated on x-axis) as compared to % of intact NMJs at end-stage in SOD1^{G93A} mice (white bars), as measured by V AchT/Btx co-localization, reveals a relationship between MMP-9 expression and axonal dieback (mean ± s.e.m, n=3-4).
Figure 2.10

<table>
<thead>
<tr>
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<th>WT</th>
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Average number of MN per section:

- WT: Orange bars
- SOD1^{G93A} end-stage: Orange bars with error bars
Figure 2.10 MMP-9 is a prospective marker for motor neurons lost at end-stage in ALS model mice.

By end-stage, although there is a 50% loss of total motor neurons at L5 in SOD1<sup>G93A</sup> mice, there is no decrease in the MMP-9<sup>neg</sup> population, in agreement with the model that they are ALS-resistant.

A-B. Immunostaining for VACht (red) to label MNs and MMP-9 (green) in age-matched WT and mSOD1 mice shows that there has been a massive loss of MMP-9-positive MNs in the mSOD1 mice by P160.

C-D. ISH confirms both the loss of ChAT-positive MNs (C) and the absence of MMP-9 RNA in cell bodies.

E. Quantification of total number of MNs (orange) and those that are MMP-9-negative (white bars). Values are mean ± s.e.m from three animals.

F. All MMP-9 expressed in spinal cord at end-stage co-localizes with microglial marker IBA-1 (red).
Figure 2.11
Figure 2.11 MMP-9-expressing motor neurons preferentially activate ER stress.

A–F. Immunostaining for MMP-9 (green) and ER stress marker phospho-EIF2α (P-EIF2α, red) in P40 spinal cord of non-transgenic (WT) and SOD1<sup>G93A</sup> mice. P-EIF2α is restricted to mSOD1 mice (compare E with B) and co-localizes with MMP-9 (F). Scale bars, 20 μm.

G. MMP-9-expressing motor neurons preferentially activate ER stress. There are significantly more SOD1<sup>G93A</sup> motor neurons co-positive for P-EIF2α and MMP-9 (yellow) than would be predicted by chance (grey bar; defined as the probability of being MMP-9 positive*probability of being P-EIF2α positive).
Figure 2.12
Figure 2.12 MMP-9 has a different expression pattern in a human’s spinal cord than in the mouse

A. H&E-stained section showing large chromatolytic motor neurons of human lumbar spinal cord from a 60 year old man who died of non-neurological disease.

B. Immunostaining of an adjacent paraffin section revealed very low levels of MMP-9 in the cell bodies of these motor neurons, but possible staining of the processes (compare the brown neuropil of B with the control secondary only stain in C).

D. Sections of spleen (from a different human patient) were used as a positive control for the MMP-9 antibody.
Chapter 3: MMP-9 is a major driver of disease in SOD1\textsuperscript{G93A} mice

Introduction

In Chapter 2, I confirmed the results of the Henderson lab microarray and extended them considerably. I showed that the expression of a single gene, \textit{Mmp9}, distinguishes different subpopulations of wild-type motor neurons and its expression is related to their vulnerability in motor neuron disease. Specifically, the most highly resistant extraocular motor pools expressed virtually no MMP-9, while pelvic muscle innervating motor pools in the DL nucleus expressed very low levels of MMP-9. In contrast, the majority of motor neurons in vulnerable pools expressed MMP-9. In the most vulnerable population of motor neurons we sampled, lumbar level alpha motor neurons, we found that MMP-9 specifically labeled the most disease prone subtype: fast MNs. We also investigated MMP-9 expression in the most commonly used mouse model of the disease: SOD1\textsuperscript{G93A} mice.

In humans, approximately 10% of all ALS cases have been identified as being familial in origin. Of these, the most prevalent identified mutations are gain-of-function point mutations in superoxide dismutase 1 (\textit{SOD1}) and hexanucleotide repeat expansions in chromosome 9 open reading frame 72 (\textit{C9orf72}) (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Rosen et al., 1993). Missense mutations in the SOD1 gene are responsible for between 14% (France) and 23% (US and Scandinavia) of familial ALS, and the G93A mutation, in which a glycine is replaced by an alanine, is frequently observed (Andersen, 2006; Siddique and Lalani, 2002).

\textit{SOD1}\textsuperscript{G93A} mice have clinical manifestations and exhibit pathology similar to human ALS (Gurney, 1994). We found a remarkable degree of positive correlation between the fraction of wild-type motor neurons expressing MMP-9 in a given region of the spinal cord or brainstem.
and the degree of susceptibility of that population to degeneration by end-stage in the SOD1$^{G93A}$ mice. Moreover, the clinical presentations in both mutant SOD1 mice and in human patients show a high correlation between FF motor neurons and vulnerability and we show that MMP-9 is selectively expressed in these fast motor neurons in mice. However, questions still remain about whether the SOD1$^{G93A}$ mouse fully recapitulates the selective vulnerability seen in patients (described in Chapter 1). We will address this question in this chapter by analyzing the amount of denervation in individual muscles of terminal SOD1$^{G93A}$ mice. Further, identifying MMP-9 as a marker for vulnerable motor neurons in disease suggests that this protein could potentially have a functional role in the degeneration of the motor neurons that express it in mutant SOD1 mice. We will test this hypothesis in this chapter by crossing $Mmp9^{-/-}$ mice and SOD1$^{G93A}$ and monitoring the disease progression in these animals, with the hypothesis that if MMP-9 makes motor neurons vulnerable to disease, then genetically depleting it will render these cells more disease-resistant.

This type of cross-breeding strategy has been used many times before to evaluate the role of a given protein, and thus often of a putative disease mechanism, downstream of mutant SOD1-induced toxicity in ALS (Turner and Talbot, 2008). The read-out for many of these studies is often a change in total lifespan for the genetically modified mutant SOD1 mice, or less often, changes in earlier disease processes such as behavioral motor deficits or selective cell death. Because the timing of degeneration is very well characterized in the SOD1$^{G93A}$ model, changes in either direction can be detected (that is, disease worsening or symptom alleviation).

For instance, cross-breeding depletion strategies have been used to examine whether impaired neurotrophin support of motor neurons might underlie their degeneration. As one example, it has been shown that ablating CNTF expression by crossing $CTNF^{-/-}$ to SOD1$^{G93A}$
mice exacerbates disease onset, leading these mice to develop ALS approximately 10 weeks earlier than their littermates that have normal CNTF levels, as measured by counting motor neurons of age-matched animals at lumbar levels (Giess et al., 2002). On the other hand, crossing knock-out mice for potentially disease-relevant proteins with SOD1\textsuperscript{G93A} mice has also led to partial disease alleviation. Human ephrin type-A receptor 4 (EPHA4), like MMP-9, is expressed by the largest alpha motor neurons. A 50% reduction in \textit{Epha4} expression in SOD1\textsuperscript{G93A} mice led to improved motor performance, reduced motor neuron degeneration, and prolonged survival by 8.5% (Van Hoecke et al., 2012).

Genetic intervention studies in mutant SOD1 mice have also attempted to dissect out critical inflammatory mediators, including MMP-9, with mixed results (Turner and Talbot, 2008). First, several studies using conditional reduction of mutant SOD1 in glia, bone marrow transplants or chimeric animals, have strongly suggested that microglia significantly influence non–cell-autonomous damage of motor neurons and contribute to ALS disease progression (Beers et al., 2006; Boillée et al., 2006a; Boillée et al., 2006b; Clement et al., 2003). Microglia are the source of NADPH oxidase, the main enzyme producing reactive oxygen species during inflammation. It has been shown to be upregulated in familial and sporadic ALS and in mutant SOD1 mice (Wu et al., 2006). Inactivation of NADPH oxidase 2 by deletion of its subunit gp91\textsuperscript{phox} extended survival in these mice from 122 days to 135 days, and also lessened other indicators of neurodegeneration, such as motor neuron and large axon loss (Wu et al., 2006). These results were supported by a later study on a mixed genetic background (Marden et al., 2007). Interestingly, deletion of TNF-\(\alpha\), a potent proinflammatory cytokine that can be synthesized and released by astrocytes, microglia, and some neurons, did not affect survival of SOD1\textsuperscript{G93A} or SOD1\textsuperscript{G37R} mice, nor did it influence motor neuron loss or gliosis (Gowing et al.,
2006). Further, there are conflicting results with regard to whether or not IL-1β affects ALS pathogenesis, with one study showing no effect of deletion on the disease (Nguyen et al., 2001), and another on a different background using a different SOD1 mutation (G93A vs. G37R) showing that IL-1β deficiency slowed disease progression but did not affect disease onset (Meissner et al., 2010). A similar discrepancy was reported for the MMP-9, with one group reporting its deletion exacerbated the disease and another later study saying its removal improved the disease course (Dewil et al., 2005; Kiaei et al., 2007).

First, as alluded to earlier, when Mmp9−/− mice on a mixed C57BL/6 129 background were crossbred to SOD1G93A on a pure C57BL/6 background, the average lifespan deceased from 139 ± 3 days in the SOD1G93A;Mmp9+/+ group to 132 ± 3 days for the SOD1G93A;Mmp9−/− animals (Dewil et al., 2005). A later study, however, reported a significant survival benefit when they crossed Mmp9−/− mice on a CD-1 background to SOD1G93A mice on the B6SJL background (Kiaei et al., 2007). In fact, they showed a very large increase in survival; in their colony, the average lifespan of SOD1G93A; Mmp9+/+ animals was 123±10 days whereas that of SOD1G93A; Mmp9−/− was 155± 12 days. They also showed significant protection of motor neurons in the spinal cord: while approximately 35% of lumbar motor neurons had degenerated by P115 in SOD1G93A; Mmp9+/+ mice, only 10% degenerated in SOD1G93A; Mmp9−/− at the same age, though this was the earliest time point that group examined. Neither group looked at early changes such as muscle denervation or differential ER stress responses in these mice, since both groups came to study MMP-9 from the perspective of its late-stage inflammatory induction. Moreover, neither recognized what we consider to be the critical site of expression: spinal motor neurons.
Therefore, despite the existence of these reports, the role of MMP-9 in motor neuron degeneration in ALS has not yet been conclusively established. Given the striking correlation of MMP-9 expression in wild-type motor neurons with their susceptibility in SOD1<sup>G93A</sup> mice reported in Chapter 2, we will assess whether or not the expression of MMP-9 by vulnerable motor neurons might contribute to their degeneration in ALS. Importantly, we focus on other metrics in addition to lifespan in order to assess the influence of MMP-9, including muscle denervation over time, motor neuron numbers, behavior, and muscle physiology. Promisingly, as Kiaei et al had done for lifespan, we report that deleting even just one allele of <i>Mmp9</i> has beneficial effects on all of these metrics and discuss clinical implications.

**Results**

**Denervation of individual extraocular muscles and lumbar sphincters is significantly delayed in ALS model mice**

After establishing that MMP-9 selectively labels the most disease susceptible populations of motor neurons, our next goal was to determine the functional contribution of MMP-9 to the disease process in ALS. Before doing so, we needed to evaluate the degree of ALS resistance of individual motor pools in SOD1<sup>G93A</sup> mice in our colony, and confirm that these animals phenocopied human patients with the sporadic form of ALS in this way. We quantified denervation of multiple muscles at end-stage by immunostaining serial longitudinal cryosections throughout the full extent of each muscle for the cholinergic synaptic marker, vesicular acetylcholine transporter (VACHT) to label motor terminals, and fluorophore-conjugated alpha-
bungarotoxin (BTX) to mark motor endplates and assessing their overlap. We observed 200-900 neuromuscular junctions per muscle.

In agreement with previous reports (Pun et al., 2006), the fast hindlimb muscle TA showed nearly complete denervation by this time, with only 13.7 ± 1.2% (mean ± standard error of mean (s.e.m), n=3) of NMJs still intact by end-stage, whereas the slow Sol was significantly more resistant with preserved innervation of approximately 50.2 ± 4.5% of endplates, again confirming previous studies (Figures 3.1A, 3.1C and 3.1E). In striking contrast, the extraocular muscles were remarkably preserved. Valdez et al. also recently reported the extraocular muscles to be collectively resistant in ALS mice (2012), but we extended this analysis by examining the eight muscles individually. Even at end-stage, the extraocular muscle superior rectus (SR) showed normal healthy synaptic morphology (Fig. 3.1B) with essentially no loss of innervation (98 ± 1 % innervated) and this resistance was shared by all eight extraocular muscles (Figure 3.1E). Likewise, innervation of the external urethral and anal sphincters (EUS and EAS) was well preserved with 85.4 ± 1.0% and 81.6 ± 4.3% of all motor endplates remaining innervated at end-stage (Figures 3.1D and 3.1E). In contrast, the innervation of muscles associated with the external genitalia, the ischiocavernosus (IC) and bulbocavernosus (BC) muscles, had significantly reduced innervation 45.1 ± 15% and 42.1 ± 14.3%, respectively. Though these numbers are much more variable than those reported for all other muscles, they generally correspond to an intermediate level of resistance, comparable to slow muscles (Figure 3.1E).

Therefore, this remarkable degree of overlap between the selectivity of muscle denervation in the SOD1G93A mouse and that in human patients with sporadic ALS strongly suggests the existence of shared mechanisms of vulnerability.
Genetic ablation of MMP-9 in ALS has strong protective effects

In order to ascertain whether MMP-9 is playing a functional role in the ALS pathogenesis, we bred $Mmp9^{-/-}$ mice to SOD1$^{G93A}$ mice, both on a pure C57BL/6 background. MMP-9 knockout mice are healthy and have a normal lifespan (Vu et al., 1998). Although they have been reported to show mild deficits in hippocampal-dependent learning (Nagy et al., 2006) and delays in wound healing (Kyriakides et al., 2009), no motor dysfunction has been found in these mice. Further, in our colony we found no muscle denervation or motor behavioral phenotype at any stage as compared to WT controls (not shown). We therefore sought to analyze how the absence of MMP-9 might affect the SOD1$^{G93A}$ phenotype.

First, we confirmed by Western blot that MMP-9 is completely absent from $Mmp9^{-/-}$ mice and that MMP-9 levels in heterozygous $Mmp9^{+/}$ mice were ~50% of those in WT mice (Figure 3.2A), and this was unchanged in combination with mutant SOD1 (Figure 3.2G and 3.2H). Further, there were no changes in SOD1$^{G93A}$ copy number or expression in the absence of MMP-9 (Figure 3.2B and 3.2J-K). We next analyzed muscle denervation in SOD1$^{G93A}$ mice lacking MMP-9 as compared to SOD1$^{G93A}$ controls. The % innervation (mean ± s.e.m.) of one fast muscle (TA) and one slow muscle (Sol) are shown in Table 3.1.

Axonal die-back occurred early in the TA muscle of SOD1$^{G93A}$ mice, as expected: 41 ± 2% (mean ± s.e.m.; n=4 animals, 1 muscle from each) of fibers were denervated by P50 and 71 ± 6% by P100 (Figures 3.3A, 3.3B and 3.3G). In contrast, Sol muscle showed no significant denervation at P50 and only 22 ± 5% loss at P100 (Figures 3.3C and 3.3H). These values are similar to those reported by Caroni and colleagues (Pun et al., 2006), and reflect the greater fraction of FF motor neurons in the TA pool. We therefore considered muscle denervation to be
a sensitive and early endpoint through which to measure disease onset and progression. Strikingly, at P50 in the TA muscle of SOD1\textsuperscript{G93A}\textsubscript{;Mmp9\textsuperscript{-/-}} mice, no denervation was detected relative to WT controls (p=0.16) and even at P100 approximately 77\% of motor endplates remained innervated, representing a 2.5-fold improvement over mSOD1 controls (Figures 3.3D, 3.3E and 3.3G). This >2.5-month delay in loss of innervation of a fast muscle is the strongest effect so far reported in ALS model mice, other than by removing the disease gene itself (reviewed in Turner and Talbot, 2008). Significant, though less dramatic given its greater resistance, protection was also observed in the slow Sol muscle (Figures 3.3F and 3.3H).

Importantly, inactivation of just a single allele of \textit{Mmp9} also conferred significant benefit in the TA. SOD1\textsuperscript{G93A}\textsubscript{;Mmp9\textsuperscript{+/-}} mice exhibited ~70\% of the protection provided by complete ablation up to P100, and equivalent protection at end-stage (Figure 3.3G). In the slow Sol muscle there was also a beneficial effect of partial \textit{Mmp9} deletion at late disease stages (Figure 3.3H). Given that we showed that MMP-9 is selectively expressed by fast motor neurons (Chapter 2, Figures 2.6-2.8), we considered a possible explanation of our data that deleting MMP-9 in SOD1\textsuperscript{G93A} mice leads motor neurons to adopt a more S-like phenotype.

In SOD1\textsuperscript{G93A} mice, denervation of the “skin-facing” compartment of the TA muscle (approximately 1/3 of whole muscle, 525 \(\mu\)m) precedes that of the “bone-facing” fibers by >20 days, suggesting that the compartments have FF and FR character, respectively (two way ANOVA, \(F=4.80, p=0.039\); Figure 3.4A; see also, Pun et al., 2006). In contrast, in the TA muscle of SOD1\textsuperscript{G93A}\textsubscript{;Mmp9\textsuperscript{-/-}} mice the two compartments became denervated at an identical rate (\(F=0.360, p=0.560\); Figure 3.4B). This suggested that in the absence of MMP-9 either FF motor neurons adopt FR-like properties, or both FF and FR tend toward the resistance of S motor neurons. We therefore compared the rate of muscle denervation by the “fast” motor neurons of
the TA pool in SOD1\textsuperscript{G93A};\textit{Mmp9}\textsuperscript{-/-} (Figure 3.3G, red points) with that observed for the mostly slow motor neurons of the neighboring Sol pool in SOD1\textsuperscript{G93A} mice with normal levels of MMP-9 (Figure 3.3H, black points). Superposition showed that there are similar levels of denervation over time except at P160, where the values are significantly different (t = -3.4, d.f. = 4, p = 0.026; Figure 3.4C). Therefore, although muscle-specific differences may also contribute, we conclude that FF and FR motor neurons lacking MMP-9 both adopt an axonal die-back phenotype comparable to that of S motor neurons. However, removal of MMP-9 does not completely convert FF motor neurons to the S subtype since we detected no change in cell body size of fast motor neurons between TA innervating MNs from age and weight-matched Mmp9\textsuperscript{-/-} and WT animals (p = 0.21), nor from all Chat\textsuperscript{+} L5 MNs in P25 SOD1\textsuperscript{G93A};\textit{Mmp9}\textsuperscript{+/-} and SOD1\textsuperscript{G93A};\textit{Mmp9}\textsuperscript{-/-} (p = 0.32).

To confirm that the preserved synapses were functional, we measured the compound muscle action potentials (CMAP; Towne et al., 2011) and also used a non-invasive measure of muscle atrophy, electrical impedance myography (Li et al., 2013). First, for CMAP, recording electrodes were inserted into the TA muscle, while another pair was used to stimulate the sciatic nerve until a maximal response was reached (Figure 3.5A). Using a computer code written by Turgay Akay that measures peak to peak CMAP amplitude but excludes the stimulus artifact (which is not produced by the nerve itself and varied from preparation to preparation, independent of the genotype of the animal), we found that from P100-P130, the CMAP in SOD1\textsuperscript{G93A} mice was reduced to 10% of that in WT controls. However, deletion of one or two copies of \textit{Mmp9} rescued up to 50% of normal values (Figure 3.5B).

In order to find a non-invasive way to detect whether the electrical activity in these muscles could be preserved, potentially at earlier time-points, in collaboration with Dr. Seward
Rutkove (Beth Israel Deaconess Medical Center) we next undertook a very preliminary study to evaluate EIM from GC muscles of 10 SOD1\textsuperscript{G93A} male mice (with or without altered \textit{Mmp9} levels) at multiple time-points, ranging from P43 to P146 (Figure 3.5C). It has been previously reported that of the parameters that EIM measures (resistance, reactance, and phase), the phase-slope provides the strongest correlation between rate of disease progression and death (Wang et al., 2011b). Therefore, this is the measure we used in our animals to determine the rate of disease progression. Though generally EIM phase decreased over time for all groups, it did not appear to be a very robust measure of electrical muscle output. Overall, the phase for the SOD1\textsuperscript{G93A};\textit{Mmp9}\textsuperscript{+/+} was 17% lower in the oldest animal of that genotype (P143) than the youngest (P43), however there was barely a difference between the phase at P80 and P100 (2% difference). In contrast, the SOD1\textsuperscript{G93A};\textit{Mmp9}\textsuperscript{−/−} at P100 was actually higher than the SOD1\textsuperscript{G93A};\textit{Mmp9}\textsuperscript{−/−} GC phase at P80 (by 15.7%). However, by P145 the phase value was similar to an age-matched SOD1\textsuperscript{G93A};\textit{Mmp9}\textsuperscript{+/+} mouse (12.2 and 12.5, respectively), and this represents a 10.7% decline from the earliest point and the latest time-point measured for this genotype.

Increasing the numbers of animals is critical to assess the outcome as the data are currently underpowered, but another explanation for variability could be that the GC muscle was evaluated, and that muscle is composed of a mixture of fiber types. Perhaps a better muscle for EIM analysis would be the TA, whose skin facing compartment has been reported to be comprised of only FF fibers (Pun et al., 2006). To further assess EIM as a method for preclinical evaluations, I also measured peak CMAP from the TA and L-GC from the same animals at the P80 and P100 time-points and correlated the 2 measures (Figure 3.5D). The P100 TA CMAP values from these animals were in line with numbers we had obtained previously.
(Figure 3.5B), confirming that the animals themselves were not the source of variability in the data set. Because the EIM measurements were done on the GC muscle, I also did CMAP recordings from that muscle and found a positive (though weaker) correlation between the 2 measures (Figure 3.5D). Therefore, the results of the CMAP study (and to a lesser extent the EIM measures), show that the neuromuscular junctions preserved by Mmp9 inactivation are functional. Corresponding to the preservation of synapses, motor neuron numbers in the lumbar spinal cord initially decreased more slowly in the absence of Mmp9, with significantly more MNs remaining at P100 in the SOD1^{G93A};Mmp9^{-/-} mice compared to SOD1^{G93A};Mmp9^{+/+} controls (p=0.003; Figure 3.6A-C). However, this protection was transient and by P160 motor neuron numbers were comparable between the two genotypes (Figure 3.6C).

We further observed significant improvements resulting from Mmp9 ablation in other functional tests that measure late changes in the mSOD1 mice, such as the rotarod (Figure 3.7A) and swim (Figure 3.7B) tests. Specifically, we consider the swim test in which a mouse is placed at one end of a tank and is timed while it swims to a platform on the other end, to be a sensitive measure of hindlimb muscle function (Raoul et al., 2005). The stage at which swim rate was reduced by 75% was delayed by 28 days in SOD1^{G93A};Mmp9^{-/-} mice, and by 18 days in heterozygotes (Figure 3.7B). The rotarod test is not specific to lumbar level evaluation and instead relies on both limb muscles and axial muscles; it also requires intact motor coordination. Still, very similar data were obtained using the rotarod test as the swim test, in terms of the timing of motor decline (Figure 3.7A).

Finally, we also consider lifespan in mSOD1 mice to be a functional readout since it is determined by the stage at which mice can no longer right themselves in 30 seconds. Median survival of SOD1^{G93A} mice in our C57BL/6 colony was 156 days. This was increased to 178
days by removal of a single allele of \( Mmp9 \) and to 195 days by complete ablation, representing a 25% increase in lifespan (Figure 3.8). Though it has been reported that survival time is reduced by 5 days in males as compared with females (Heiman-Patterson et al., 2005), we do not see this in our colony in which there is no significant differences between males and females for any of the 3 genotypes examined (\( \text{SOD1}^{G93A}, Mmp9^{-/} \), p=0.85; \( \text{SOD1}^{G93A}, Mmp9^{+/} \), p=0.61; \( \text{SOD1}^{G93A}, Mmp9^{+/+} \), p=0.80). Interestingly, the MMP-9-dependent differences in survival data are similar to those reported by Kiaei et al. (2007; see Discussion), even though our studies were performed on a different, congenic genetic background, and even though the lifespan of our \( \text{SOD1}^{G93A} \) mice was longer. Taken together, these data show that MMP-9 plays a role in ALS pathogenesis.

**Discussion**

MMP-9 was initially identified as a potential therapeutic target for ALS based on our analysis of molecular diversity between motor neurons that are vulnerable or resistant to ALS. In Chapter 2 we showed that MMP-9 expression prior to disease onset defines the subset of motor neurons that are destined to die which are also most vulnerable in human patients: the fast motor neurons. In this chapter, our data show that MMP-9 is more than just a marker of susceptible populations; it plays a major role in motor neuron degeneration in ALS model mice.

Importantly, we used a variety of metrics beyond just survival time to assess potential changes in the \( \text{SOD1}^{G93A} \) mice with one or both \( Mmp9 \) alleles deleted. Early reports initially described the disease as “presymptomatic” in the \( \text{SOD1}^{G93A} \) transgenic mouse model prior to
P90, when significant motor neuron loss from the lumbar spinal cord is detected coincident with the onset of tremors and hindlimb weakness. From this age, progressive paralysis of the hindlimbs during the symptomatic phase was reported to culminate in their near complete paralysis followed by death when the animal can no longer right itself after being placed on its back (Chiu et al., 1995; Gurney, 1994). However, later studies demonstrated that the die-back of axons from the muscle end-plates occurs much earlier and there is a dramatic decline in the number of functionally intact motor units during the asymptomatic phase of disease (Azzouz et al., 1997; Fischer et al., 2004; Hegedus et al., 2007; Pun et al., 2006).

We chose to focus primarily on this early axonal dieback because 1) it is the earliest reported morphological change in mSOD1 mice; 2) it has been shown to reflect the differential vulnerability of FF vs. FR vs. S MN subtypes (Frey et al., 2000); 3) denervation is a phenomenon that occurs with near-identical specificity in human patients (Fischer et al., 2004) and 4) it has direct clinical relevance since it is the defining event in the onset of muscle paralysis. We find that measuring muscle denervation is extremely robust; indeed, the % innervation in the TA is nearly invariable between littermates regardless of sex at a very early time point (P50; see Table 3.1, Figure 3.3) and a power analysis showed that only 3-4 animals are needed to detect a treatment effect (G*POWER version 3.1, two-tailed test, $D = 3.15$, noncentrality parameter = 4.45, critical $T = 2.45$, actual power =0.96). Using this measure, we showed a spectacular delay in muscle denervation (~80 days) in the SOD1$^{G93A;Mmp9^{-/-}}$ mice, compared to controls. We further coupled this with CMAP measurements and behavioral assessments to show a real preservation of muscle strength.

Although there are many advantages to using an assessment of the morphology of the NMJ as an early outcome, one drawback is that it requires that the animal be killed. We had
hoped to find a less invasive measure, and so explored the use of EIM. In EIM, a weak, high-frequency electrical current is passed across a set of electrodes overlying a muscle of interest in an anesthetized mouse and the consequent surface voltages are measured. These voltages provide a measure of muscle pathology including muscle atrophy and the presence of fat and connective tissue within the muscle (Rutkove, 2009). Human studies have shown that the EIM 50 kHz phase value is highly reproducible between individuals and very sensitive to decline in ALS and the method is being optimized to transition to preclinical animal studies (Nie et al., 2006). Unfortunately, in a small sample, we found that EIM was not very sensitive to differences in rates of decline and atrophy in mice. We speculate that this is because the prototype that we used for measurements was designed for use on the GC muscle. Future studies in other muscles, especially the TA, will be of interest to further explore the potential use of the method as a part of a preclinical screening protocol. With these considerations, for the majority of the experiments we will describe in the next chapter of this thesis, we used NMJ morphology as our primary outcome to evaluate the effect of different treatments.

Our denervation data in TA and Sol muscles are in line with previous electrophysiological studies and fiber type analyses that support the sequence of degeneration from FF to FR to S (Hegedus et al., 2007; Kanning et al., 2010). Further electrophysiological studies also suggested that an initial switch in motor unit phenotype from FF to FR may precede the loss of FF motor axons (Hegedus et al., 2008). Our data imply that removing MMP-9 may facilitate a motor neuron subtype conversion, as we have shown that there are no differences in susceptibility between deep and superficial layers of the TA in the SOD1$^{G93A}$, $Mmp9^{-/-}$ mice (Figure 3.4B). The resistance of FR and S motor units may reflect their high sprouting capacity, which may allow them to reinnervate motor endplates (Frey et al., 2000). Therefore, future
studies to assess whether MMP-9 contributes to ALS pathology by preventing regenerative axon sprouting or rather by actively triggering denervation will be important.

The potential role of MMP-9 in preventing axonal sprouting will be discussed in more detail in Chapter 4, but we believe it has some role, based on motor neuron numbers and behavioral data presented in this chapter. Specifically, by P160 both SOD1\textsuperscript{G93A}; Mmp9\textsuperscript{+/c} and SOD1\textsuperscript{G93A}; Mmp9\textsuperscript{c/c} mice have lost similar numbers of motor neurons, but yet at this time most SOD1\textsuperscript{G93A}; Mmp9\textsuperscript{+/+} have already died whereas all SOD1\textsuperscript{G93A}; Mmp9\textsuperscript{c/c} are still alive and many are able to swim and remain on the rotarod. This suggests that MMP-9 deletion likely promotes axonal sprouting and an increase in the average motor unit size.

Our model of the involvement of MMP-9 in ALS differs significantly from that of Kiaei et al (2007), despite the fact that our survival data are very similar. As mentioned earlier, this group explored the contribution of MMP-9 to cytokine-mediated late-stage pathology in mSOD1 mice on a mixed genetic background. Though we confirm that their findings are valid on a congenic background, in contrast to (Dewil et al., 2005), our data differ from theirs importantly in terms of cell-type and timing. Because they believed that MMP-9 was upregulated late in the disease process and was not expressed by healthy motor neurons, they limited their analysis to postnatal day 115 (equivalent to P145 on our C57BL/6 background according to Kiaei et al.). Further, their expression data for the absence followed by upregulation of MMP-9 is weak: they show a tightly cropped gel zymography image which lacked a protein ladder or protein controls and also performed immunostaining using an MMP-9 antibody from Santa Cruz Biotechnology, Inc. that is infrequently used for immunohistochemistry and receives bad user reviews on that company’s website. The authors went on to use more unquantified, low-resolution immunostaining to conclude that MMP-9 could contribute to the pathogenesis of ALS by
facilitating the cleavage of TNF-α and related proinflammatory cytokines and anchorage-dependent detachment of cells from the extracellular matrix. Though it is possible that MMP-9 may indeed play such roles during the final, catastrophic phase of degeneration during which we showed that MMP-9 is also expressed by invading microglia (Figure 2.10F), this takes place ~100 days later than the early role in TA axonal dieback that we present here. We will further explore the timing and relevant cell types involved in MMP-9’s contribution to disease in the next chapter.

Though understanding MMP-9’s mode of action is critical, our data showing the strong protection conferred by removal of MMP-9 already suggest that MMP-9 is an exciting candidate therapeutic target. This is particularly true given that we saw very significant protective effects after deleting just one allele of Mmp9 (thereby reducing MMP-9 levels by ~50%). Completely removing MMP-9 from human patients is not feasible, but there are already tools available that can reduce MMP-9 activity (Overall and López-Otín, 2002) and controlling MMP-9 expression is an area of active research (Yan and Boyd, 2007). However, at this point it is still not known by what mode of action MMP-9 is working to destabilize nerve-muscle contacts. Rational drug design requires a greater understanding of the role of MMP-9 in ALS. Therefore, the experiments described in Chapter 4 will begin to address this.
Figures

**Figure 3.1**

![Image of Figure 3.1 showing immunofluorescence staining of muscle fibers in different regions.](image-url)
Figure 3.1 Extraocular and pelvic sphincter motor units show selective resistance in ALS model mice.

A-D. Overlap of VACHT-positive motor terminals (green) with acetylcholine receptors stained using α-bungarotoxin (BTX, red) as an indicator of innervated motor endplates in end-stage SOD1<sup>G93A</sup> mice. (A) The fast TA muscle exhibits marked denervation, whereas the slow Sol remains partially innervated (C). (B) The extraocular muscle <i>superior rectus</i> (SR) and (D) the <i>external urethral sphincter</i> (EUS) show nearly complete preservation of motor units. Scale bar, 20 μm.

E. Percentage of intact neuromuscular junctions in indicated limb (green), extraocular (pink) and pelvic (blue) muscles of end-stage SOD1<sup>G93A</sup> mice (mean ± s.e.m from 3 animals). The percentage of intact neuromuscular junctions in age-matched wild-type controls ranged from 97 to 100% for all muscles (n=3) and is indicated by the grey-shaded region. Muscles (top row): <i>levator palpebrae</i> (LP), <i>medial rectus</i> (MR), <i>inferior rectus</i> (IR), <i>inferior oblique</i> (IO), <i>retractor bulbi</i> (RB), <i>superior oblique</i> (SO), <i>lateral rectus</i> (LR), <i>ischiocavernosus</i> (IC), <i>external anal sphincter</i> (EAS), <i>bulbocavernosus</i> (BC). Motor neurons (bottom row): lumbar spinal cord (L), oculomotor nucleus (III), trochlear nucleus (IV), abducens nucleus (VI), dorsolateral and dorsomedial components of Onuf’s nucleus (DL and DM).

_Collaboration with A. Kaplan._
Figure 3.2

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Figure 3.2 MMP-9 and mutant SOD1 levels in mSOD1 mice crossed to MMP-9 knockouts

A. Western blotting reveals that MMP-9 levels in the heterozygote are 44% of the wildtype control. Densitometry values were normalized using α-tubulin.

B. No changes are observed in SOD1G93A copy number in the absence of MMP-9. Quantitative real-time PCR (qPCR) data using the Delta Ct method are shown.

C-K. Immunostaining for ChAT (red), MMP-9 (blue), and mSOD1 (green) at P40 in L5 spinal cord of indicated genotypes. Overall motor neuron numbers are similar but MMP-9 intensity per cell reflects the null or heterozygote genotype. Note complete absence of MMP-9 staining in the SOD1G93A;Mmp9−/− spinal cord, demonstrating the specificity of the MMP-9 antibody. No difference in intensity of mSOD1 staining was noted between three Mmp9 genotypes.
Table 3.1 Percent muscle innervation at indicated time-points of SOD1\textsuperscript{G93A} hindlimb muscles with 0, 1, or 2 copies of \textit{Mmp9}

\textit{Tibialis Anterior}

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\textit{ Soleus}

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<td>71.0 ± 6.9</td>
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Table 3.1 Percent muscle innervation over time

Percent muscle innervation at indicated time-points of SOD1^{G93A} hindlimb muscles with 0, 1, or 2 copies of Mmp9. Numbers are mean ± S.EM, n=3-4 animals with one muscle from each animal counted. Data are plotted in Figure 3.3G and H.
Figure 3.3

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**BTX VACH T**

| MmpB+/+ GB3A | A    | B    | C    |

| MmpB-/- GB3A | D    | E    | F    |

**G**

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% innervated NMUs

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**H**

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% innervated NMUs

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Figure 3.3 Genetic ablation of MMP-9 markedly delays muscle denervation in SOD1\textsuperscript{G93A} mice.

A-F. Neuromuscular junctions in TA and Sol muscles in SOD1\textsuperscript{G93A} mice that are either wildtype or null-mutant for \textit{Mmp9}. At P50 in the fast TA muscle of a normal SOD1\textsuperscript{G93A} mouse, partial denervation is already apparent (A) but neuromuscular junctions are preserved in a SOD1\textsuperscript{G93A};\textit{Mmp9}\textsuperscript{−/−} littermate at the same age (D). Even more marked preservation is observed at P100 (compare E to B). In the slow Sol at P100 denervation is only partial in mice wildtype for \textit{Mmp9} (C) and this loss is blocked by ablation of \textit{Mmp}-9 (F). Scale bars, 20 \(\mu\)m.

G-H. Morphological and functional evaluation of four different genotypes, color-coded in the same way in both panels: SOD1\textsuperscript{G93A};\textit{Mmp9}\textsuperscript{+/+} (black; +/+)\textsuperscript{+/-}, SOD1\textsuperscript{G93A};\textit{Mmp9}\textsuperscript{−/+} (blue; +/−), SOD1\textsuperscript{G93A};\textit{Mmp9}\textsuperscript{−/−} (red; −/−), and non-transgenic (gray, WT). (G) Denervation of TA muscle is 50\% complete by 63 days in normal SOD1\textsuperscript{G93A} mice but delayed by \(\sim80\) days by removal of either one or both alleles of \textit{Mmp9}. Values represent means ± s.e.m of one muscle from each of 3-4 animals per genotype (*** \(p<0.001, **p< 0.01\)). (H) Denervation of slow Sol muscle occurs 90 days after that of the TA, but is further delayed by 50 days in the absence of MMP-9. Values represent means ± s.e.m of one muscle from each of 3-4 animals per genotype (***\(p<0.001, **p< 0.01\)). See also Table 3.1.
Figure 3.4 In the absence of MMP-9, fast motor neurons adopt “slower” properties

A. In SOD1<sup>G93A</sup> mice, innervation of the superficial, skin-facing compartment (black points) precedes that of the deep, bone-facing fibers (blue points) by >20 days. The difference in the mean values of FF vs. FR is greater than would be expected by chance after allowing for effects of differences in time (P = 0.039).

B. Both subcompartments of the TA of SOD1<sup>G93A;Mmp9<sup>−/−</sup></sup> mice become denervated at an identical rate (P = 0.560).

C. There are no significant differences at early time-points in the amount of innervation of the “slow” Sol muscle of mSOD1 mice (red points) and the “fast” TA of the SOD1<sup>G93A;Mmp9<sup>−/−</sup></sup> mice (blue points). Therefore, FF and FR motor neurons both adopt a behavior that is comparable to that of S motor neurons. Data replotted from Figure 3.3G and H.
Figure 3.5

A

B

C

D
Figure 3.5 Measuring electrical activities in SOD1$^{G93A}$ mice shows that removing MMP-9 rescues muscle function

A-B. Evoked CMAPs in the TA muscle after stimulation of the sciatic nerve in mice of the indicated genotypes. (A) Individual traces show the stimulus artifact (s.a.) followed by the M-wave (whose maximum peak-to-peak value is used to calculate the CMAP) and H-wave. (B) CMAP measurements from groups of the indicated genotypes aged between P105-130 were compared with age-matched non-transgenic controls (WT) by one way ANOVA. Results are shown as mean ± s.e.m. (n= 3-4 animals per genotype); * p<0.05.

Collaboration with T. Akay.

C. EIM measures (50 kHz phase) for 3 animals of each genotype measured at 4 different timepoints; SOD1$^{G93A}$;Mmp9$^{+/+}$ (black filled circles), SOD1$^{G93A}$;Mmp9$^{+-}$ (blue open circles), SOD1$^{G93A}$;Mmp9$^{-/-}$ (red triangles).

D. Correlation plot of EIM 50 kHz phase with peak CMAP amplitude from the GC muscle from 5 animals at P80 and P100 shows a moderate positive correlation between the two measures, $R^2=0.437$.

Collaboration with S. Rutkove.
Figure 3.6 Motor neuron numbers in the spinal cord initially decrease more slowly in the absence of Mmp-9

A, B. Motor neurons visualized using ChAT (green) at the L5 level of the spinal cord of P100 SOD1\textsuperscript{G93A};Mmp9\textsuperscript{+/+} and SOD1\textsuperscript{G93A};Mmp9\textsuperscript{-/-} littermates.

C. Direct counting of motor neurons at L5 from P40 to end-stage shows a significant delay in loss in the absence of MMP-9 (n=3-5; p=0.003 at P100).
Figure 3.7
Figure 3.7 Deletion of a single or both Mmp-9 alleles confers significant functional benefit in SOD1<sup>G93A</sup> mice

Behavioral evaluations of four different genotypes, color-coded in the same way in both panels: SOD1<sup>G93A</sup>;Mmp9<sup>+/-</sup> (black; +/+), SOD1<sup>G93A</sup>;Mmp9<sup>++</sup> (blue; +/-), SOD1<sup>G93A</sup>;Mmp9<sup>-/-</sup> (red; -/-), and non-transgenic (gray, WT).

A. Latency to fall from an accelerating rotarod from P40 until mice were no longer able to remain on the rotarod for 5 seconds. SOD1<sup>G93A</sup>;Mmp9<sup>-/-</sup> mice performed better than controls from P70 onward as assessed by one-way ANOVA at each time-point (***, p<0.001, **, P<0.01; *, p<0.05). Mmp9 heterozygotes showed improved performance from P150 (p<0.001).

B. Mmp9 deletion delays motor impairment in a swimming task that reflects hindlimb function. Swim rate declines rapidly after P124 in SOD1<sup>G93A</sup>;Mmp9<sup>++</sup> mice (n=7). From P145 onward, decline is significantly delayed in both SOD1<sup>G93A</sup>;Mmp9<sup>++</sup> (n=8, p=0.03) and SOD1<sup>G93A</sup>;Mmp9<sup>-/-</sup> (n=10, p=0.005) mice.

Data were obtained in collaboration with G. Choe.
Figure 3.8
Figure 3.8 Deletion of a single or both Mmp-9 alleles extends lifespan in SOD1\textsuperscript{G93A} mice

Kaplan–Meier plot showing the cumulative probability of survival of SOD1\textsuperscript{G93A};Mmp9\textsuperscript{−/−} (\(n = 12\), red line), SOD1\textsuperscript{G93A};Mmp9\textsuperscript{+/−} (\(n = 19\), blue line) and SOD1\textsuperscript{G93A};Mmp9\textsuperscript{+/+} (\(n = 16\), black line) on a pure C57BL/6 background (log-rank test= 49.2, p<0.001). Lifespan is prolonged 14\% by deletion of one Mmp9 allele and 25\% by deletion of both.
Chapter 4: Role of MMP-9 in motor neuron degeneration

Introduction

In Chapter 2 I reported that MMP-9 is selectively expressed in vulnerable MNs at two levels: in susceptible motor pools (trigeminal, facial, L5 MNs, RDL) and also in susceptible MN subtypes (fast α-MNs) within spinal motor pools. Further, we showed that ER stress and UPR, measured by p-EIF2α positivity, are selectively induced in MMP-9+ motor neurons, in agreement with their FF/FR identity. In Chapter 3, we found that MMP-9 is a major driver of disease in SOD1G93A mice. Genetic deletion delays TA muscle denervation by approximately 80 days, delays functional motor declines in the accelerating rotarod, a swim test, and TA CMAP, and increases lifespan by 25%. These exciting findings raise many interesting questions that we will begin to address with experiments presented in this chapter. For example, in what cell type is MMP-9 inducing its pathological effect and when is this happening? Is MMP-9 sufficient to induce axonal degeneration? What is the role of enzymatic activity versus non-catalytic binding? Can we identify a candidate mechanism (or parallel mechanisms) downstream of MMP-9 that fit into an ER stress-relevant pathway? And finally, what are the potential clinical implications of this research?

First, it may be useful to briefly review pathways that have already been implicated in the toxicity of mutant SOD1. The exact molecular pathway remains unknown, but it is widely agreed that the pathological effects of SOD1 mutations are not the result of a loss of dismutase activity, but rather from gain-of-function toxic effects. This theory is supported by several lines of evidence. For example, mice that are completely deficient for SOD1 develop normally and show no overt motor deficits out to 6 months in age, though they did exhibit marked
vulnerability to motor neuron loss after axonal injury (Reaume et al., 1996). This suggests that SOD1 is not necessary for normal motor neuron development and function but is required under physiologically stressful conditions following injury. Moreover, survival time is the same in mice carrying a mutant SOD1 transgene (tgSOD1\textsuperscript{G85R}) on a normal mouse background compared with the same transgene on a SOD1\textsuperscript{+/−} background, demonstrating that survival is independent of mouse SOD1 loss of function (Bruijn et al., 1998). Further, motor neuron degeneration occurs in transgenic mice overexpressing several different mutant forms of SOD1, irrespective of residual dismutase activity (Gurney, 1994). In motor neurons, mutant SOD1 toxicity may result from various effects, including oxidative stress, accumulation of intracellular SOD1-positive aggregates, mitochondrial dysfunction and defects in axonal transport (Rothstein, 2009). A unifying theory has been presented in which these multiple stressors converge to generate instability of proteostasis through vicious cycles of cell stress and protein misfolding (Saxena and Caroni, 2011).

Because mutant SOD1 is ubiquitously expressed, identifying the cell type(s) in which it acts to trigger degeneration has been a very interesting and productive area of research. Studies support the notion that mutant SOD1 in both MNs and non-neuronal cells such as microglia and astrocytes contributes to the disease process in vivo (Papadimitriou et al., 2010). First, when expression of mutant SOD1 is restricted to either motor neurons (neurofilament light chain or Thy1 promoters) or astrocytes (GFAP promoter), transgenic mice develop normally and do not experience spontaneous motor deficits with increasing age (Gong et al., 2000; Lino et al., 2002; Pramatarova et al., 2001), except at a very late age with extremely high levels of neuronal mutant SOD1 expression (Jaarsma et al., 2008). Second, Clement et al. (2003) generated chimeric mice that are mixtures of normal cells and cells expressing SOD1 mutations and found that as the
percentage of wild type cells increases, chimeras show progressively less severe disease. Further, wild type motor neurons show pathology in chimeras where they are next to SOD1 mutant non-neuronal cells. These results argue that motor neuron degeneration in SOD1 mutants is not cell-autonomous, but rather is in some way mediated by surrounding, non-neuronal cells (Clement et al., 2003). Finally, removal of mutant SOD1 expression by Cre recombinase-mediated excision from either astrocytes or microglia slowed the disease progression and extended survival (Boillée et al., 2006b; Wang et al., 2011a; Yamanaka et al., 2008). Therefore, the SOD1 mutation acts in both cell-autonomous and non-cell-autonomous manners.

Although we originally identified MMP-9 because of its expression in susceptible motor neurons themselves, it remained possible that MMP-9 from other cell types is the critical actor in the degenerative process (Kiaei et al., 2007). Thus, in order to determine the involvement of motor neuron derived-MMP-9, we will use adeno-associated virus (AAV) vectors to selectively silence MMP-9 in motor neurons and look for an effect on TA muscle innervation. AAV vectors are small, replicative defective viruses that exhibit a number of properties advantageous for gene therapy in the central nervous system. First, depending on age of administration and AAV serotype used, the majority of AAV vector transduction can occur in neurons where it is possible to obtain long-term, stable gene expression with very little accompanying toxicity after only one injection (McCown et al., 1996). AAV serotypes are variations of the virus resulting in capsid protein motifs that are not recognized by the same neutralizing antibodies. The capsid of AAV determines the method of entry into the host cell (Weinberg et al., 2013). Since the earliest work with AAV2, multiple other AAV serotypes that are either naturally occurring or laboratory designed have been investigated. Further, distinct from the tropism inherent to different AAV vector serotypes, promoter selection, virus purification method, and route of delivery to the
animal all significantly influence the pattern and longevity of neuronal transduction (Blankinship et al., 2006). Finally, another factor that influences transgene expression is the conversion from a single-stranded (ss) DNA genome that is transcriptionally inactive into a double-stranded (ds) template. The ssDNA to dsDNA conversion is the rate-limiting step involving either the de novo synthesis of the second-strand DNA or the annealing of the plus and minus strands from two separate viral particles coinfecting into the same cell (Ferrari et al., 1996; Fisher et al., 1996). To accelerate transduction, AAV vectors have been developed that package a self-complementary dsDNA genome (Wang et al., 2003).

In the last decade, AAV vectors have successfully been used to manipulate CNS function through a wide variety of approaches including expression of foreign genes, expression of endogenous genes, expression of antisense RNA and expression of RNAi (Weinberg et al., 2013). For motor neuron disease in particular, there have been some exciting recent studies that have used AAV gene transfer. Several groups have investigated the use of different AAV serotypes to target motor neurons in the spinal cord after intramuscular, intraneural, intraparenchymal, or intrathecal injections (Hollis et al., 2008; Snyder et al., 2011; Towne et al., 2008). Further, two different groups showed that administration into the facial vein 1 day after birth of self-complementary AAV serotype 9 expressing survival motor neuron (SMN) protein in spinal muscular atrophy model mice resulted in a substantial extension of life span in these animals (Foust et al., 2010; Valori et al., 2010). Similar results were reported after intracerebroventricular (i.c.v.) and intraspinal injection with AAV8 (Passini et al., 2010; Samaranch et al., 2012). Therefore, AAV vectors can serve as powerful tools for preclinical studies and are very promising as tools for gene therapy.
In this chapter, we will use different strategies to ask when MMP-9 is playing a role in ALS, where it is acting, and by what molecular pathway it exerts its effect. We test different AAV vectors to find the optimal serotype to overexpress or silence Mmp9 specifically in motor neurons. We will also use pharmacological inhibitors of MMP-9 to explore the role of enzymatic activity in triggering motor neuron degeneration.

Results

MMP-9 is required for the full activation of ER stress in the ALS disease pathway

In Chapter 2, I showed there was a selective and early induction of UPR, as measured by p-EIF2α, in MMP-9-positive motor neurons at P40. To determine the relative positions of MMP-9 and the UPR in the degenerative pathway, we next examined p-EIF2α staining at P40 and P50 in SOD1\(^{G93A}\) mice that were null or wildtype for Mmp9 at P40 and P50. We observed a strong increase in p-EIF2α staining between P25 and P40 in the SOD1\(^{G93A}\);Mmp9\(^{+/+}\) mice (Figure 4.1 A-B), which was significantly dampened in the SOD1\(^{G93A}\);Mmp9\(^{-/-}\) littermates. In fact, at P40, only <25% of the normal number of motor neurons stained for p-EIF2α, and this fraction was even lower at P50 (Figures 4.1C and 4.1G). By P75, when TA muscle denervation has begun even in SOD1\(^{G93A}\);Mmp9\(^{-/-}\) mice, the number of motor neurons stained for p-EIF2α had risen to 5.8 ± 0.9 (n=2 animals) per section (Figure 4.2B), suggesting that Mmp9 deletion delays, but does not indefinitely prevent, the onset of ER stress. Indeed, at this time point, p-EIF2α staining in the spinal cords of SOD1\(^{G93A}\);Mmp9\(^{+/+}\) littermates was widespread and detectable in almost all motor neurons as well as neighboring ChAT-negative cells (Figure
4.2A). Thus MMP-9 very likely acts as one major factor upstream of activation of the ER stress response in a majority of fast motor neurons. This makes MMP-9 the earliest known biochemical intermediate in motor neuron degeneration triggered by mutant SOD1.

**Acute motor neuron knockdown of Mmp9 also has protective effects**

We next sought to determine the cell type in which MMP-9 exerts its effects on motor neuron degeneration. Since no conditional mutants for Mmp9 have been reported, we decided to use the targeting properties of AAV vectors. We first tested different AAV serotypes and routes of administration to find an optimal combination to specifically target motor neurons but not other cell types. The transduction profiles of various vectors are summarized in Table 4.1.

Along with colleagues in the Henderson lab, various AAV serotypes encoding the reporter green fluorescent protein (GFP) were delivered to WT pups at either P1 (for i.c.v. or i.v. administration) or P4 (for i.m. administration). The animals were sacrificed at P25 and the transduction efficiency of the AAV administration in their spinal cords was assessed by examining the sites of GFP fluorescence. We found that the AAV6:cmv-EGFP generated by the Aebischer group at EPFL resulted in the most specific motor neuron transduction. However, AAV6 from UNC did not transduce MNs. Further, no self-complementary AAV (from Guangping Gao at UMMS) exclusively transduced MNs when delivered i.c.v.; that is, we either saw no transduction, or transduction of both MNs and other neural cell types. The 2 best self-complementary serotypes (following i.c.v. administration) were scAAV6.CB6.eGFP (Figure 4.3A) and scAAVrh8.CB6.eGFP (Figure 4.3B), though neither were as motor neuron-selective as the single-stranded AAV6 from the Aebischer lab (Figure 4.3C-F).

Also, we observed a high level of toxicity after i.c.v. administration with some serotypes (most toxic: scAAVrh39 and scAAVrh43) and the toxicity by i.c.v administration was highly
correlated to the amount of diffusion around the site of injection for that same serotype in the intramuscular injection experiment. For example, for those 2 most toxic serotypes, all 3 of the animals treated with each had severe brain dissolution at the time of the perfusion and had bilateral whole body fluorescence following a unilateral TA injection. Consequently, following these analyses, AAV6 from EPFL was selected for future motor neuron targeting studies.

When administered into the cerebral ventricles in neonatal mice, AAV6 engenders stable gene expression in 50-60% of motor neurons throughout the rostrocaudal extent of the spinal cord, in addition to some dorsal neurons and fiber tracts and sensory ganglia (Figure 4.3C and not shown). However, AAV6 does not efficiently transduce brainstem motor nuclei (Figure 4.4A-C) and so we limited our analyses to the lumbar level of spinal cord. Moreover, despite efficient MN transduction, no obvious fluorescence was observed in muscles or the livers of the animals, suggesting that this route of administration has no or limited transduction of cells in the periphery. Further, in agreement with previous reports (Snyder et al., 2011; Towne et al., 2009; Towne et al., 2011), when AAV6-cmv.GFP is injected into a single skeletal muscle (i.m.) at P4, only the muscle and the corresponding motor pool are labeled (Table 4.1). This allowed us to compare the effects of reducing MMP-9 in motor neurons + CNS + sensory neurons (i.c.v.) with those of knockdown in motor neurons + muscle + proprioceptive sensory neurons (i.m.).

We first determined shRNA sequences that were optimal for knockdown of MMP-9 (not shown). These were incorporated, together with a mismatch control, into AAV6 vectors (Figure 4.5A). Virus was injected either at P1 (i.c.v.) or P4 (i.m.) and the effects on MMP-9 expression in vivo were analyzed at P50. Injection i.c.v. gave a significant but partial (20%; p=0.03) overall reduction in numbers of MMP-9+ motor neurons, and a stronger (50%; p=0.0003) reduction in MMP-9hi (Figure 4.5F). The incomplete removal of MMP-9 by this
approach can be explained by the fact that not all motor neurons were infected and, of those that were, that some did not show complete knockdown (Figures 4.5B and 4.5C). Overall there was a strong correlation between GFP expression and lack of MMP-9 expression, as with i.m. injection into TA (Figures 4.5D and 4.5E).

Despite the fact that MMP-9 reduction was only partial, we observed strong protective effects of AAV6 shRNA administered by either route (Figures 4.5G-I). At P50, when SOD1\textsuperscript{G93A} mice show <60% innervation of TA motor endplates, the corresponding amount for i.c.v. animals was significantly higher: 88 ± 2.2% (Figure 4.5I). This trend continued out to P100; though only 2 SOD1\textsuperscript{G93A} animals were studied at this time-point, their muscle innervation was significantly higher than untreated controls (mean ± st. dev: 59.8 ± 14% vs. 29.2 ± 11 %, p=0.049). For i.m. injection into TA, we compared shRNA to MMP-9 with the mismatch control injected on the other side of the same animals. Protection at P50 was strong, and specific to shMMP9 (Figures 4.5G to 4.5I). Likely reflecting the incomplete knockdown of MMP-9, both i.c.v. and i.m. routes gave protection indistinguishable from that observed in mSOD1 mice that were heterozygotes for MMP-9. Given that both i.c.v. and i.m. routes led to comparable protection, and that i.c.v. administration in neonatal mice selectively infects motor neurons and some dorsal spinal and proprioceptive sensory neurons and injection into a single skeletal muscle labels only the muscle and the corresponding motor pool and proprioceptive neurons, these experiments implicated MMP-9 in motor neurons or possibly proprioceptive sensory neurons. However, since proprioceptive neurons do not express MMP-9 even in ALS mice (Figure 2.4), our results identify MMP-9 in motor neurons themselves as the trigger of degeneration.

**MMP-9 is sufficient to induce degeneration in fast motor neurons of ALS mice**
Given that removing MMP-9 from motor neurons rescues muscle innervation, we next asked whether MMP-9 was alone sufficient to trigger axonal die-back. We first tested the effects of MMP-9 in ALS-resistant motor units. An AAV6 vector expressing full-length *Mmp9* cDNA was injected at P4 into the extraocular muscles of WT and SOD1<sup>G93A</sup> mice (n=4 each), resulting in stable expression of MMP-9 in a significant fraction of oculomotor neurons throughout adulthood, at levels comparable to those in FF motor neurons (Figure 4.6A and 4.6C). However, we detected no increase in denervation of extraocular muscles even at P150, with or without mutant SOD1 (Figure 4.1B). Similar results were observed at P100 in left vs. right Sol muscles infected in the same way (p=0.20) (Figure 4.6B and 4.6E). Thus, simple expression of MMP-9, or co-expression with mutant SOD1, in a neuron is not sufficient to trigger its degeneration, suggesting that other elements of the pathway also need to be present. Accordingly, when we overexpressed MMP-9 in the fast TA motor pool of SOD1<sup>G93A</sup> mice, denervation of this vulnerable muscle was further accelerated (Figure 4.6B and 4.6D), but there was no effect in WT controls. When we compared the MMP-9<sup>+</sup> populations from the injected side versus the uninjected side of spinal cord from the SOD1<sup>G93A</sup> mice that had received unilateral injection of AAV6.MMP9 into their TA, we found no difference in the total number of MMP-9<sup>+</sup> cells per ventral horn (9.7 ± 1.0 vs. 9.4 ± 1.1 MNs/side, p=0.18). However, we found a large increase in the number (2.4-fold) of MMP-9<sup>hi</sup> cells.

We therefore asked whether MMP-9 is sufficient to restore vulnerability to TA motor neurons rendered resistant by *Mmp9* deletion. AAV6-MMP-9 was injected into one TA muscle of a neonatal SOD1<sup>G93A; Mmp9<sup>-/-</sup></sup> mouse, while either no virus (n=2) or AAV6-GFP (n=2) was injected into the contralateral TA as a control (Figure 4.7A). By P50, the control muscle remained fully innervated whereas ~22% of the NMJs became denervated in the injected muscle.
(Figure 4.7B-G). There was a strong inverse correlation between the number of transduced motor neurons (MMP-9\(^+\)) and the % innervation in the TA (R\(^2\)=0.85, Figure 4.7E). Overall, therefore, within the cellular context of fast motor neurons expressing mutant SOD1, the rate of degeneration is proportional to the level of MMP-9. However, activation of the pathway for axonal die-back requires neuronal subtype-specific elements in addition to MMP-9 and SOD1.

**MMP-9 fits into an ER stress-relevant pathway**

Besides showing that MMP-9 is sufficient to induce muscle denervation in fast motor pools, using AAV6.MMP9 to selectively express MMP-9 unilaterally in the TA motor pool of SOD1\(^{G93A}\);Mmp9\(^{-/-}\) mice provides an *in vivo* system for further studies on the role of MMP-9 in ALS. Given that we showed that MMP-9 was required for the phosphorylation of EIF2α to occur (Figure 4.1), we decided to use this system to situate MMP-9 within a known pathway involved in MN vulnerability and induction of ER stress. Briefly, it was previously shown that cultured mSOD1 motor neurons, but not WT motor neurons, are susceptible to cell death induced by Fas-ligand and nitric oxide (NO) (Raoul et al., 2002). Further, elevated calcium levels and loss of calreticulin (CRT) are critical determinants of this susceptibility and are upstream of ER stress and death (Bernard-Marissal et al., 2012). It was also shown that this loss of CRT occurs selectively in fast motor neurons, as Sol motor neurons showed CRT levels that were indistinguishable between WT and SOD1\(^{G93A}\) mice, whereas the levels of CRT in TA MNs were reduced by 60% (Bernard-Marissal et al., 2012). Therefore, we decided to ask whether CRT levels and MMP-9 levels are inversely correlated on a cell-by-cell basis in mSOD1 spinal cord and if SOD1\(^{G93A}\);Mmp9\(^{-/-}\) mice have normal CRT levels. We indeed found that the average CRT immunofluorescence of MNs at P50 in uninjected SOD1\(^{G93A}\);Mmp9\(^{-/-}\) L5 mice was the same as in age-matched WT animals (p=0.45). In SOD1\(^{G93A}\);Mmp9\(^{-/-}\) mice (n=3) with MMP-9 virally
expressed in the left TA motor pool, there was a significant down-regulation in CRT immunofluorescence on the injected side at P50 (Figure 4.8A-C). When the injected side is further divided into MMP9-positive MNs and MMP9-negative MNs versus MMP-9-negative MNs from the uninjected side (65-100 MNs in each category), it is clear that the MMP-9-positive MNs themselves have the lowest CRT levels (Figure 4.8D). Further, there was no difference between MNs transduced with AAV6.GFP and adjacent non-transduced MNs (p=0.92), suggesting that AAV6 alone is not sufficient to change CRT levels. Therefore, these results combined with those of Bernard-Marissal et al. (2012) suggest that while Fas activation leads to activation of nNOS and NO production in all mSOD1 motor neurons, only those that express MMP-9 manifest a decrease in CRT levels and an induction of ER stress.

The enzymatic activity of MMP-9 interferes with axonal sprouting of FF MNs, but is likely not involved in their initial dieback

Neither the results of the experiments with transgenic animals nor AAV-mediated knock-down indicate by what mode MMP-9 leads to motor neuron degeneration. MMP-9 has been shown to mediate its effects through both its proteolytic activity and through non-enzymatic processes that rely on hemopexin domain binding (Van den Steen et al., 2006; Vandooren et al., 2013b), and it is important to distinguish between these potential modes of action in motor neuron degeneration. Further, it is also unclear whether MMP-9 acts within the spinal cord or at the neuromuscular junction. A previous study using an orally-delivered broad inhibitor of MMPs found only a modest 13-day survival benefit in mSOD1 mice (Lorenzl et al., 2006), but this may have reflected insufficient CNS exposure. A commercially available inhibitor (MMP inhibitor I, Calbiochem), which is among the most potent and specific for MMP-9, was therefore
administered centrally (2 µg i.c.v., daily) to SOD1\textsuperscript{G93A} mice from P55 (the earliest time point at which stereotactic administration is feasible) to P75 (Figure 4.9A-B). The inhibitor caused a significant delay in TA muscle denervation as compared to controls (Figure 4.9C) and reduced levels of ER stress in the ventral horn of SOD1\textsuperscript{G93A} animals at P75, compared to vehicle-treated controls (Figure 4.9D and 4.9E). This indicates that enzymatic activity is at least partially involved in the degenerative process and this likely occurs at the level of the spinal cord, either directly on fast motor neurons or on neighboring cells and synapses. Encouragingly, these results show that MMP-9 inhibition initiated even after the start of the degenerative process can confer significant protection.

We next wanted to compare the protection conferred by inhibitors delivered before axonal dieback or, as before, after the initial muscle denervation has already occurred. Because we found that before P50 our mice were often too small (<16 g) with skulls that were too thin to accommodate a head mount that is required for chronic i.c.v. administration (Figure 4.9B), we treated 2 groups of mice with SB-3CT, an irreversible inhibitor of gelatinase activity (selective for both MMP-9 and MMP-2) that had been shown to cross the blood-brain barrier at a dose of 25 mg/kg, i.p. (Gooyit et al., 2012). Two groups of animals were treated with SB-3CT or vehicle (10 % DMSO) from either P35-P50 (Group 1) or from P55 to P75 (Group 2) (Figure 4.10A). There were initially 5 SOD1\textsuperscript{G93A} animals in Group 2 that were receiving the inhibitor treatment, but we observed serious side effects in 3 of these including one with a massive skin lesion (likely from reduced wound healing as a result of the gelatinase inhibition) and 2 from apparent gastrointestinal blockages (which were excluded from the study following weight loss). Thus, by the P75 time-point, only 2 SOD1\textsuperscript{G93A} animals had received the full treatment schedule, which is not a large enough group to detect changes in muscle innervation and more animals are
needed to really understand the effect of the treatment at P75. However, both of those animals had a greater amount of innervation in the TA muscles than any of the controls at P75, though with such a small sample size there was not a significant overall difference between the groups (avg ± SD, 72.9 ± 11 % vs. 57.1 ± 7.1 %, p=0.13) (Figure 4.10C), and the % innervation was very similar to the animals that had been treated during the same timeframe with MMP-9 Inhibitor I (Figure 4.9C). Moreover, these values at P75 were much greater than the amount of innervation reported in untreated animals at P50 or P60 (Figure 4.10C). Therefore, these preliminary data suggest that inhibiting MMP-9 allowed for a greater amount of reinnervation to occur.

The unexpected side effects were managed better in Group 1, which received drug holidays after every 5 days of treatment, and fewer total injections than Group 2 (13 vs. 21 injections). In these younger animals, there was no difference between the groups that received 2 weeks of vehicle treatment versus 2 weeks of the treatment with inhibitor from P35 to P50 (p=0.91, n=3 animals per group, Figure 4.10). Because of the low numbers of animals in these studies, and because attempts to quantify the amount of inhibition that the treatment caused by either gel or in situ zymography have failed thus far (Figure 4.11 and not shown), interpreting these data require caution. However, a tentative model could be that the enzymatic activity of MMP-9 inhibits reinnervation, but is not involved in early pathology that results in the initial FF axonal die-back, given that SB-3CT had no effect on TA innervation when delivered between P30 and P50. Future studies will be required to support this model, especially with regard to understanding the role of MMP-9 in early pathology. However, the effect of MMP-9 inhibitors on muscle innervation at relatively late stages in disease may be very promising as a therapy for patients who are diagnosed after muscles have already lost a significant amount of innervation.
Discussion

Overall, these data significantly extend our understanding of the role of MMP-9 in ALS. We have shown that MMP-9 in motor neurons themselves contributes to their degeneration. We have also shown that MMP-9 is acting early, and that it is upstream of CRT down-regulation and the induction of ER stress. Since to date CRT down-regulation and ER stress are the earliest biochemical events reliably linked to the mSOD1 degeneration pathway, these data suggest that MMP-9 is a very early element in the degeneration pathway downstream of SOD1. Further, we show that the effects are mediated by its enzymatic activity after initial muscle denervation, but possibly not before. Therefore, the results of these experiments strengthen the rationale for the use of MMP-9 reduction as a therapeutic option for ALS patients.

First, we used AAV6 as an important tool for many of the experiments presented in this chapter. We began by comparing different serotypes and routes of administration and found that there are important differences in cell transduction between vectors that greatly impact their utility. Of all those tested, we found that AAV6:cmv-EGFP had the most efficient and selective motor neuron transduction when administered into the ventricles at P1. Why should this particular vector differ so markedly from the other AAV6 vectors that we tested? We speculate that it is one or a combination of the following 3 potential reasons. First, promoter choice certainly plays an important role in transduction of different cells. The AAV6 vectors from the Aebischer lab and UNC used CMV and the scAAV6 from UMMS used the CBA promoter. Following our tests with these, it was reported that relative to the CMV promoter, the CBA promoter expressed poorly in MNs in the brainstem and spinal cord, resulting in an underestimate of the number of MNs targeted by AAV9 (Gray et al., 2011). This agrees well with our data. However, they also report that while the CMV promoter provides rapid and strong
transgene expression, it is prone to diminish significantly over time in certain cell types. In contrast, we found stable, high expression in motor neurons with either AAV6:cmv-EGFP or AAV6.cmv.MMMP9 in motor neurons to the latest time point examined (P150, Figure 4.6C).

Another difference may arise from the purification method used in virus preparation, as it has been argued that the method of purification may have a greater impact on cell type tropism than packaging (Summerford and Samulski, 1999; Zolotukhin et al., 1999). Guangping Gao (UMMs) and David Dismuke (UNC) both used a classic CsCl₂ sedimentation method for purification, whereas the Aebischer lab used a newer “HiTrap” heparin column-based method. Finally, the titers differed for the 3 different AAV6 vectors sampled. We compared the different serotypes by normalizing to injection volume, but it may have been better to compare a fixed vector genome dose. One of the reasons that we chose volume over titer, is that there is currently not a standard way to titer AAV preparations and different groups contest each other’s results (David Dismuke, UNC, personal communication). It is possible that the AAV6 vector from UNC would have performed as well as the AAV6 from the Aebischer group if a larger volume had been injected.

We also saw significant toxicity with several of the vectors from UMMS that we tested, but saw no equivalent toxicity with the vectors from EPFL or UNC. The difference here is likely that those vectors from Guangping Gao at UMMS used a self-complementary (sc) vector design which has a very different vector biology than the native single strand (ss) vector design which the other groups used. Sc vector has a much faster onset and higher level of transgene gene expression as compared to ss vector (5- to 10-fold higher and at least 7-10 days faster, which may lead to more and rapid accumulation of toxic EGFP) (Guangping Gao, UMMS, personal communication). Ss vector needs to be converted into double stranded vector genome before it
starts expression, whereas sc vector is a double stranded vector and will start expression immediately after entering the cells and uncoated. This ss to double-strand conversion process is the rate-limiting step for AAV transduction and many ss AAV genomes get lost in this process (Ferrari et al., 1996). Sc vector bypasses this process and get much better transduction efficiency (Wang et al., 2003). However, for a potentially neural toxic protein such as EGFP (Liu et al., 1999), this increased efficiency likely was detrimental.

Therefore, we chose to use AAV6 from the Aebischer group at EPFL for experiments to ascertain the cell type from which the MMP-9 is derived that influences motor neuron degeneration. We found that AAV6 shRNA to MMP-9 administered either i.c.v. or i.m. protected muscle innervation to the same extent as that seen in the heterozygote knockout. The only cell types transduced by both routes of administration are motor neurons and proprioceptive neurons, and the latter did not express MMP-9. We therefore conclude that it is the MMP-9 expressed by vulnerable motor neurons themselves that drives their die-back or, alternatively, prevents their regeneration during the cycle of denervation-reinnervation that precedes paralysis (Schaefer et al., 2005). However, since MMP-9 is a secreted extracellular enzyme, this does not preclude actions at sites in the immediate vicinity of the motor neurons, such as afferent synapses or the neuromuscular junction.

In this chapter, we have also presented clear genetic evidence that MMP-9 is required for the full activation ER stress, as previously defined in an elegant series of experiments by the Caroni lab (Saxena et al., 2009). Beyond delaying the upregulation of p-EIF2a in MNs, we have also shown that down-regulation of CRT is prevented at P50 in SOD1^{G93A} mice with Mmp9 deleted. We went on to show that introducing MMP-9 to a specific motor pool in the SOD1^{G93A};Mmp9^{-/-} mice not only induces muscle denervation in the TA, but also causes a
decrease in CRT immunofluorescence on a cell-by-cell basis in their spinal cords. By what mechanism is MMP-9 acting to induce these early changes? Our experiments suggest that MMP-9 could be upstream of the phosphorylation of EIF2α, but do not distinguish between enzymatic and non-enzymatic activities of the protein. Our preliminary study with the pharmacological activity inhibitor (SB-3CT) delivered from P35 to P50 suggests that at this stage MMP-9 is not inducing pathology through its activity. Though drawing solid conclusions from these data is premature, there is support in the literature for how both MMP-9 proteolysis and/or integrin binding could possibly induce these changes.

MMP-9 has been shown to act in an integrin β1-dependent manner to influence NMDA surface trafficking and enhance NMDA receptor function (Michaluk et al., 2009). This in turn activates nNOS (Bredt and Snyder, 1989) and thereby the production of nitric oxide (NO). Another potential source of NO is a distinct feedback loop in ALS mice through which activation of the Fas death receptor by Fas ligand (FasL) leads in a motor neuron-specific manner to transcriptional upregulation of neuronal NOS (Raoul et al., 2002). Interestingly, MMP-9 itself has been shown to regulate the release of FasL (Xu et al., 2011). Further, NO can activate MMP-9 through both direct and indirect mechanisms including S-nitrosylation (Gu et al., 2002), creating a potential vicious cycle. Increased levels of NO can lead through multiple routes to enhanced phosphorylation of EIF2α, for example by reductions in levels of calreticulin (Bernard-Marissal et al., 2012). Therefore, a role for MMP-9 upstream of ER stress is consistent with our current knowledge of its mode of action.

This model suggests that concurrent with ER stress activation, high levels of NO will also convert proMMP9 to the active form which, when deregulated, has been shown to have other deleterious effects in the CNS (Yong, 2005). One of many possible scenarios of how the
enzymatic activity of MMP-9 could contribute to disease, is that APP is a known substrate of MMP-9 (Backstrom et al., 1996). N-terminal fragments of APP can activate the death receptor DR6, which has been shown to be involved in axonal retraction at the neuromuscular junction (Nikolaev et al., 2009). Moreover, inactivation of the APP gene in SOD1<sup>G93A</sup> mice delays muscle denervation without improving motor neuron survival or lifespan (Bryson et al., 2012). Since the effects of MMP-9 deletion appear to be stronger than those of either APP inactivation or inhibition of ER stress (Saxena et al., 2009), it is likely that MMP-9 acts upstream of a series of parallel pathways, each of which contributes to a specific element of the ALS phenotype. The inhibitor data also support a model involving multiple pathways—in that we found that enzymatic activity is not involved in early pathology (by P50), but inhibiting it has protective effects later (P75) (Figure 4.10C).

These experiments with the pharmacological inhibitors are exciting, but must be interpreted with caution as the SB-3CT study has a number of potential drawbacks. First, SB-3CT was chosen because it has been documented to cross the blood-brain barrier following systemic injection (Gooyit et al., 2012) and has been used successfully in several studies of MMP pathology in the central nervous system (Bell et al., 2012; Gu et al., 2005b; Guo et al., 2010). However, SB-3CT, unlike MMP-9 Inhibitor I, acts on both MMP-9 and MMP-2 by directly binding to the zinc in the gelatinase catalytic site (Brown et al., 2000). Given that MMP-2 is not expressed in adult motor neurons (Figure 2.3), we consider potential effects on motor function more likely to be the result of the MMP-9 inhibition. Still, the side effects that we saw in 3 of the 5 SOD1<sup>G93A</sup> animals in Group 2 (Figure 4.10A) are consistent with pan-gelatinase inhibition in terms of problems with wound healing (Gill and Parks, 2008) and gastrointestinal problems (Medina and Radomski, 2006; Mott and Werb, 2004). Interestingly, side effects like
these have also been reported in human patients treated with MMP2/9 inhibitors, including diarrhea and loss of appetite (Hoekstra et al., 2001; Siller and Broadie, 2012).

Another drawback is that we have not yet been able to determine the actual amount of inhibition that had occurred following drug treatment, because our attempts to quantify the amount of MMP-9 activity in specific tissues have failed so far. The standard way to measure net MMP-9 activity is using gel zymography or high resolution in situ zymography. In gel zymography, electrophoretic methods are used for measuring proteolytic activity. The method is based on a sodium dodecyl sulfate gel impregnated with a protein substrate which is degraded by proteases. Later staining reveals sites of proteolysis as clear or white bands on a dark background, and thus visualization is based on protease activity (Leber and Balkwill, 1997). Because MMPs are produced and secreted as zymogens that require proteolytic cleavage to be activated, separation by size can differentiate between active and pro-MMP-9 (as well as MMP-2). In media obtained from cell culture, it is relatively easy to detect MMPs and indeed we were able to detect high amounts of MMP-9 activity using conditioned media from human macrophage cultures (not shown). However, the extraction and analysis of MMPs from tissue homogenates has proven to be much more difficult, despite the strong expression data that indicate presence of the protein in a given cell or region. There could be a few reasons that we have had such difficulty. First, the amount of MMPs in tissues is about 50 times lower than those found in conditioned medium (Woessner, 1995). Secondly, MMPs are difficult to extract from tissues, and it is never certain whether all MMPs are extracted. Moreover, MMP-9 is often tightly bound to the ECM (Snoek-van Beurden and Von den Hoff, 2005; Woessner, 1995). We have tried various ways to circumvent some of these problems, including using Sepharose beads to concentrate MMP-9 in homogenates, but so far activity detection has not been robust even in
positive controls like spleen or placental samples. Further, while this technique would be useful for measuring endogenous MMP activity (good for testing the hypothesis that MMP-9 becomes pathologically active after increases in NO and ER stress), it would not detect activity reduction by the pharmacological inhibitors which may become detached from the zinc during the tissue homogenization or SDS treatment. This has been reported to even be true for irreversible inhibitors (Hadler-Olsen et al., 2010).

Another disadvantage of tissue extraction is the preclusion of the localization of MMPs, an important aspect for a protein that we have shown to label only a susceptible subpopulation (Vandooren et al., 2013a). Specific MMPs or TIMPs localized in a small part of the tissue may not be detected because of their dilution in the entire tissue extract (Yan and Blomme, 2003). A better technique for the localization of enzymatic activity is fluorescent in situ zymography. In this approach, a very tightly packed, dye-quenched (DQ-) fluorescein-gelatin becomes fluorescent only after its quenching is relieved by the action of the gelatinase. Pilot attempts to use in situ zymography to localize MMP-9 activity in spinal sections in our laboratory have yielded results with very poor resolution and unreliable fluorescence even in a negative control or on slides pre-treated with very high concentrations of inhibitors (Figure 4.11). Several other groups have reported difficulty with this technique (Christopher Overall, UBC, personal communication) and many papers that use it including Kiaei et al (2007), show cropped images and do not include important controls. Finally, we have also tried antibodies that were designed to recognize either pro or active forms of the enzyme but we discontinued their use after we found they both gave signals in Mmp9−/− tissue. Therefore, experiments to quantify activity levels have all proven unsuccessful. Alternative strategies for future work on the role of the enzymatic activity of MMP-9 will be discussed further in Chapter 6.
Therefore, though questions remain about the exact molecular mechanism by which MMP-9 exerts its pathologic effects, we have gained some valuable insights in this chapter. Our data have shown that the cell-autonomous role in motor neurons is at least partially mediated through its enzymatic activity in the spinal cord and that MMP-9 plays an early role, upstream even of the phosphorylation of EIF2α. Further, we believe that the strategies to explore the relationship of MMP-9 and ALS that we have described in this and previous chapters (including AAV knockdown and overexpression and systematic analyses of muscle denervation) will be of great use in studying other candidate therapeutic targets. In fact, in Chapter 5 we will present expression and functional data from experiments with other potential targets.
Figure 4.1

<table>
<thead>
<tr>
<th></th>
<th>SOD1G93A;Mmp9+/+</th>
<th>SOD1G93A;Mmp9−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>P25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-eIF2α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-eIF2α, NeuN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P40</td>
<td></td>
<td></td>
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</tbody>
</table>

![Image of cellular images and bar graph](image-url)

Bar graph showing the number of eIF2α MNs per section over age (days) for WT, SOD1G93A;Mmp9+/+, and SOD1G93A;Mmp9−/− groups.
Figure 4.1 MMP-9 is required for the full activation of ER stress during the degenerative process in SOD1\textsuperscript{G93A} motor neurons

A–F. The normal abrupt appearance of P-EIF2α at P40 in SOD1\textsuperscript{G93A} motor neurons (Saxena et al., 2009) is severely damped in the absence of MMP-9. Scale bar, 20 μm.

G. Induction of ER stress at P40 is detectable in SOD1\textsuperscript{G93A};Mmp9\textsuperscript{−/−} mice (red) but occurs in >4-fold fewer motor neurons than in normal mSOD1 mice (black). ***p<0.01 compared to p-EIF2α-positive cells in normal mSOD1 mice.
Figure 4.2

<table>
<thead>
<tr>
<th>P75, SOD1&lt;sup&gt;G93A&lt;/sup&gt;</th>
<th>Mmp9&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Mmp9&lt;sup&gt;-/-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
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</tbody>
</table>

**MMP-9, p-EF2α**
Figure 4.2 Mmp9 deletion delays, but does not indefinitely prevent, the phosphorylation of EIF2α

A-B. Immunostaining for MMP-9 (green) and ER stress marker phospho-EIF2α (p-EIF2α, red) in P75 spinal cord of SOD1\textsuperscript{G93A} (A) and SOD1\textsuperscript{G93A};Mmp9\textsuperscript{-/-} mice (B). Though p-EIF2α immunopositivity is widespread by P75 in the SOD1\textsuperscript{G93A} spinal cord, it is restricted to motor neurons in the SOD1\textsuperscript{G93A};Mmp9\textsuperscript{-/-} mice in approximately 5.8 MNs/ventral horn at this time-point.
Table 4.1 Comparison of AAV Vectors for motor neuron gene delivery

<table>
<thead>
<tr>
<th>serotype</th>
<th>Route of admin</th>
<th>source</th>
<th>titer</th>
<th>general comments</th>
<th>% MNs transduced</th>
<th>experimenter</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV6:cmv-EGFP</td>
<td>ICV</td>
<td>EPFL</td>
<td>$10^{13}$ vg/ mL to $3.9 \times 10^{14}$ vg/ml</td>
<td>Very nice motor neuron specific transduction</td>
<td>59 ± 9%</td>
<td>Krista Spiller</td>
</tr>
<tr>
<td>AAV6:cmv-EGFP</td>
<td>IM</td>
<td>EPFL</td>
<td>$10^{13}$ vg/ mL to $3.9 \times 10^{14}$ vg/ml</td>
<td>Successful transduction of corresponding motor pool and proprioceptive neurons</td>
<td>Varied by injection (for TA from 30-65 MNs)</td>
<td>Krista Spiller</td>
</tr>
<tr>
<td>scAAVrh8.CB6.eGFP</td>
<td>ICV</td>
<td>UMMS</td>
<td>$1.3 \times 10^{13}$ GC/mL</td>
<td>3 animals perfused, all appeared grossly normal and were included in the analysis</td>
<td>Lumbar: 36 ± 3%</td>
<td>Krista Spiller</td>
</tr>
<tr>
<td>scAAVrh8.CB6.eGFP</td>
<td>IM</td>
<td>UMMS</td>
<td>$1.3 \times 10^{13}$ GC/mL</td>
<td>Lots of diffusion; the whole leg was fluorescent from the foot to the pelvis; visibly yellow even without microscope.</td>
<td>none</td>
<td>Krista Spiller</td>
</tr>
<tr>
<td>scAAV6.CB6.eGFP</td>
<td>ICV</td>
<td>UMMS</td>
<td>$1 \times 10^{13}$ GC/mL</td>
<td>3 animals perfused, but 1 had blood between brain and skull and a fair amount of necrosis and was not used</td>
<td>Lumbar: 53 ± 5%</td>
<td>Krista Spiller</td>
</tr>
<tr>
<td>scAAV6.CB6.eGFP</td>
<td>IM</td>
<td>UMMS</td>
<td>$1 \times 10^{13}$ GC/mL</td>
<td>The whole leg was fluorescent from the foot to the pelvis; visibly yellow even without microscope.</td>
<td>Very few (less than 10 total)</td>
<td>Krista Spiller</td>
</tr>
<tr>
<td>scAAV9.CB6.EGFP</td>
<td>IV</td>
<td>UMMS</td>
<td>$1.5 \times 10^{13}$ GC/mL</td>
<td>Periphery very well transduced (most organs green); in SC, very high DRG and dorsal horn</td>
<td>Lumbar: 32 ± 11%</td>
<td>Krista Spiller</td>
</tr>
<tr>
<td>Virus</td>
<td>Route</td>
<td>Lab</td>
<td>Dose</td>
<td>Description</td>
<td>Transduction</td>
<td>Authors</td>
</tr>
<tr>
<td>----------------------</td>
<td>-------</td>
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<td>------</td>
<td>--------------------------------------------------</td>
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</tr>
<tr>
<td>AAV6/TR-eGFP</td>
<td>ICV</td>
<td>UNC</td>
<td>$3 \times 10^{12}$ vg/mL</td>
<td>some fluorescence, but no MNs transduced</td>
<td>none</td>
<td>Krista Spiller</td>
</tr>
<tr>
<td>scAAV7.CB6.eGFP</td>
<td>ICV</td>
<td>UMMS</td>
<td>$2.3 \times 10^{13}$ GC/mL</td>
<td>3 animals perfused, all appeared grossly normal</td>
<td>none</td>
<td>Ya Dai/Hai Li</td>
</tr>
<tr>
<td>scAAV8.CB6.eGFP</td>
<td>ICV</td>
<td>UMMS</td>
<td>$1.5 \times 10^{13}$ GC/mL</td>
<td>3 animals perfused, all appeared grossly normal</td>
<td>none</td>
<td>Ya Dai/Hai Li</td>
</tr>
<tr>
<td>scAAV9.CB6.eGFP</td>
<td>ICV</td>
<td>UMMS</td>
<td>$3.5 \times 10^{13}$ GC/mL</td>
<td>3 animals perfused, all appeared grossly normal</td>
<td>none</td>
<td>Ya Dai/Hai Li</td>
</tr>
<tr>
<td>scAAV2.CB6EGFP RGB</td>
<td>ICV</td>
<td>UMMS</td>
<td>$1 \times 10^{13}$ GC/ml</td>
<td>not quantified</td>
<td>Significant tropism to ventral horn at cervical and thoracic levels</td>
<td>Dima Yudin</td>
</tr>
<tr>
<td>scAAV5.CB6EGFP</td>
<td>ICV</td>
<td>UMMS</td>
<td>$2.03 \times 10^{13}$ GC/ml</td>
<td>not quantified</td>
<td>GFP positive cells are scattered over the sections with no preference for ventral horn</td>
<td>Dima Yudin</td>
</tr>
<tr>
<td>scAAV6.2CBEGFP</td>
<td>ICV</td>
<td>UMMS</td>
<td>$1 \times 10^{13}$ GC/ml</td>
<td>not quantified</td>
<td>Very low transduction. Almost no GFP positive cells.</td>
<td>Dima Yudin</td>
</tr>
<tr>
<td>scAAVrh39.CB6.EGFP</td>
<td>ICV</td>
<td>UMMS</td>
<td>$1.2 \times 10^{13}$ GC/ml</td>
<td>All animals had severe liquefactive necrosis</td>
<td>n/a</td>
<td>Justin Lee</td>
</tr>
<tr>
<td>scAAVrh43.CB6.EGFP</td>
<td>ICV</td>
<td>UMMS</td>
<td>$3 \times 10^{13}$ GC/ml</td>
<td>All animals had severe liquefactive necrosis</td>
<td>n/a</td>
<td>Justin Lee</td>
</tr>
</tbody>
</table>

**IM**= intramuscular injection into the TA (2 µL virus injected into the TA @ P3);  
**ICV**= intracerebroventricular administration (unilateral injection of 4 µL virus + 1 µL dye @ P1),  
**IV**= intravascular administration into the facial vein (100 µL injected @ P1)  
**scAAV**= self-complementary adeno-associated virus  
**AAV**= single-stranded adeno-associated virus
Summary of the transduction profile in the spinal cord after administration with several AAV serotypes generated in different labs and administered by different routes. AAV6:cmv-EGFP generated by the Aebischer lab at EPFL resulted in the most specific motor neuron transduction and was selected for future motor neuron targeting studies.

Data were obtained in collaboration with Ya Dai, Justin Lee, Hai Li, and Dima Yudin.
Figure 4.3

scAAV6.CB6.eGFP

scAAVrh8.CB6.eGFP

AAV6.CMV.GFP

Vacht

GFAP

IBA-1

GFP

D

E

F
Figure 4.3 Different AAV serotypes have very different transduction profiles following a single intracerebroventricular injection at P1.

A-B. The self-complementary viruses generated by Guangping Gao (UMass) transduce some motor neurons, as well as other cell types. Representative images of lumbar level ventral horns from animals treated with scAAV6.CB6.eGFP (A) and scAAVrh8.CB6.eGFP (B) with motor neurons labeled with ChaT (red) and transduced cells stained with anti-GFP (green).

C-F. AAV6 from the Aebischer group (EPFL) selectively infects motor neurons and not other cell types as shown here by representative immunostained cryosections of adult lumbar cord. GFP staining overlaps with VAChT-labeled motor neurons (D), but not GFAP-labeled astrocytes (E) or IBA-1 labeled microglia (F).
Figure 4.4

AAV6.CMV.GFP

<table>
<thead>
<tr>
<th>ChaT GFP</th>
</tr>
</thead>
</table>

A

B

C
Figure 4.4 AAV6 delivered i.c.v does not efficiently transduce brainstem motor nuclei

A-C. Representative immunostained cryosections of adult oculomotor (A), trigeminal (B), and facial (C) nuclei show that very few motor neurons (ChAT, red) were transduced following an i.c.v injection with AAV6.CMV.GFP at P1. Note very few green cells.
Figure 4.5 Gene silencing of MMP-9 in motor neurons delays muscle denervation.

A. Schematic of viral construct and procedures. (Top) AAV6 expressing the MMP-9 silencer sequence TRC31231 under the H1 promoter together with GFP under the CMV promoter (AAV6:shMmp9). (Middle) i.c.v injections (n=3 for each genotype) were performed at P1. (Bottom) Injections into TA muscle (mismatch control on contralateral side) were performed at P4 (n=2/3 animals per genotype).

B-E. Reduction of MMP-9 at the single-cell level in P50 WT mice following AAV6:shMmp9 administration by each route. Transduced α-MNs, visualized by colocalization of GFP fluorescence (green) and NeuN immunostaining (blue), stained for MMP-9 (red). Asterisks: GFP⁺ α-MNs negative for MMP-9; arrowheads: less frequent GFP⁺ α-MNs that retain MMP-9 immunoreactivity. Scale bars, 20 μm.

F. AAV6:shMMP9 (i.c.v) leads to a 19% reduction in the total number of MMP9⁺ motor neurons (*p=0.03), and a 47% reduction in the numbers of MMP-9 hi (p=0.0003).

G-H. MMP-9 silencing delays muscle denervation in TA. Images from contralateral TA muscles of the same SOD1 G93A animal show normal levels muscle denervation on the side injected with AAV6:mismatch (H), but significant preservation following injection with AAV6:shMMP9 (G).

I. Comparable effects of AAV6 vectors administered by either central or peripheral routes implicate motor neuron MMP-9 as the trigger of muscle denervation in mSOD1 mice. Degree of TA innervation at P50 in SOD1 G93A mice following genetic reduction in MMP-9 levels (gray bars, data from Figure 5G) or viral silencing performed i.c.v. (purple) or i.m. (red). Knockdown by either route confers protection similar to that afforded by heterozygote deletion of Mmp9 (dotted line). The contralateral mismatch control is shown in black. Values are means ± s.e.m (n= 3-4 animals per treatment group).
**Figure 4.6**

### A

![Diagram of a mouse and gene expression](image)

### B

<table>
<thead>
<tr>
<th></th>
<th>TA</th>
<th>Sol</th>
<th>SR</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV6:Mmp9</td>
<td><strong>60</strong></td>
<td><strong>80</strong></td>
<td><strong>80</strong></td>
</tr>
<tr>
<td>Control</td>
<td>40</td>
<td>60</td>
<td>80</td>
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</table>

*Significance: **p < 0.01*  

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<th>Sol</th>
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<tr>
<td>ChAT MMP-9</td>
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</tr>
</tbody>
</table>

**SOD1<sup>G93A</sup>, i.m. injections**
Figure 4.6 MMP-9 induces degeneration in fast, but not slow, motor neurons of ALS mice

A. Schematic of viral construct and procedures. (Top) AAV6 expressing full-length mouse MMP-9 under CMV promoter. (Bottom) Injections into TA or Sol muscles or intraorbital injections (control AAV6-GFP injection contralateral side) were performed at P4 (n=3-4 animals per genotype).

B. Comparison of AAV6-MMP9-injected muscles (red bars) with contralateral control muscles (black bars) reveals that introduction of MMP-9 is not sufficient to accelerate denervation in resistant motor pools - Sol or SR - of SOD1<sup>G93A</sup> mice. In contrast, further increasing levels of MMP-9 in a susceptible pool (TA) does accelerate denervation.

C-E. Immunostaining for MMP-9 of the oculomotor nuclei of a P150 SOD1<sup>G93A</sup> mouse whose extraocular muscles had been infected at P4 with AAV6-MMP9 confirms successful transduction and stable, high levels of MMP-9 (red) in infected motor neurons (ChAT<sup>+</sup>, green) (C). High levels of MMP-9 (white) in motor neurons are observed in P100 (E) and P50 (D) SOD1<sup>G93A</sup> spinal cord sections following injection of AAV6-MMP9 into the Sol or TA muscles at P4.

*Data were collected in collaboration with S. Deivasigamani.*
Figure 4.7

**SOD1^{G93A};Mmp9^{−/−}, TA injections**

A

Left (AAV6.MMP9)
Right (control)

MMP-9

B

Left TA

C

Right TA

D

% innervated MNs

AAV6/MMP9
control

E

% innervated MNs

MMP-9 + MNs, L3

\[ f(x) = -0.24x + 92.3 \]

\[ R^2 = 0.85 \]
Figure 4.7 MMP-9 is sufficient to restore vulnerability to TA motor neurons rendered resistant by *Mmp9* deletion

A. Immunostaining for MMP-9 (red) on a transverse spinal cord section of a P50 *SOD1*<sup>G93A</sup>;<*Mmp9<sup>−/−</sup></sup> mouse that was injected at P4 with AAV6-MMP9 into its left TA muscle.

B-C. Neuromuscular junctions in both TAs of a *SOD1*<sup>G93A</sup>;<*Mmp9<sup>−/−</sup></sup> in which MMP-9 was virally expressed in the left TA motor pool, but the contralateral side remained without MMP-9. The right TA (C) is still almost completely preserved, whereas significant denervation has occurred on the left side (B).

D. *Mmp9* significantly restores vulnerability to the TA muscle (red bar), compared to the control contralateral side (black bar). **p<0.01

E. The number of motor neurons that were transduced by a single intramuscular injection of AAV6.MMP9 is proportional to the amount of denervation that has occurred in that muscle.
Figure 4.8

P50 SOD1<sup>693A</sup>;Mmp9<sup>−/−</sup> with AAV.MMP9 in TA

A. MMP-9+ side
B. MMP-9<sup>−/−</sup> side

C. Mean Pixel Intensity (a.u.)
- Injected side (AAV6.MMP-9)
- Unjected side

D. Mean Pixel Intensity (a.u.)
- MMP-9+
- MMP-9-
- Unjected side (AAV6.MMP-9)

**
Figure 4.8 Virally introducing MMP-9 precipitates a decrease in calreticulin levels in TA motor neurons rendered resistant by Mmp9 deletion

A. Representative images from the left and right side of a single spinal cord section from a P50 SOD1<sup>G93A</sup>;<sup>Mmp9<sup>-/-</sup></sup> mouse that had been injected into one TA at P4 with AAV6.MMP9 stained with CRT (green) and MMP-9 (red).

B-C. There is a significant decrease in CRT immunopositivity in all neurons of the injected side versus the control side (B). Further, when the injected side is divided into MMP-9 positive and MMP-9 negative categories, it is clear that the lowest CRT levels are in the MMP-9 positive cells themselves (mean ± sem, n=3 animals) **p<0.01

Data were collected in collaboration with S. Deivasigamani.
Figure 4.9

A

P00  P50  P75

stereotactic surgery  Daily i.c.v. injections  perfusion

B

C

100

% innervated NMJs

80

60

40

20

0

WT + 1h  inhibitor  vehicle  sham

SOD1<sup>G93A</sup>

**  n.s.

D

SOD1<sup>G93A</sup>, daily i.c.v. injections

<table>
<thead>
<tr>
<th></th>
<th>vehicle</th>
<th>MMP-9 Inhibitor I</th>
</tr>
</thead>
</table>

D: P-Elf2a

E:
Figure 4.9 Central MMP-9 inhibition confers significant protection

A. Experimental timeline. Animals were treated with MMP-9 Inhibitor I (2 µg, i.c.v) daily from P55 to P75.

B. Picture of a mouse receiving i.c.v drug administration into a chronically implanted cannula.

C. MMP-9 Inhibitor I protected the TA muscle from denervation in SOD1\textsuperscript{G93A} mice at P75 (purple bar), compared with a vehicle-treated group (red bar, n=5-6 per treatment). The inhibitor did not have an effect on muscle innervation in WT controls (grey bar), nor was there a difference between SOD1\textsuperscript{G93A} mice treated with vehicle and the animals that received the SHAM surgery but no drug (black bar).

D-E. The normal high levels of p-EIF2α (white) at P75 in SOD1\textsuperscript{G93A} motor neurons are significantly reduced by treatment from P55 to P75 with MMP-9 Inhibitor I.
Figure 4.10

A

P35  P50  P55  P75
i.p. injections, 5 days on, 1 day holiday (13 inj. ttl)
Daily i.p. injections

Group 1

Group 2

perfusion

B

Group 1

% innervated NMJs

<table>
<thead>
<tr>
<th></th>
<th>VEH</th>
<th>SB-3CT</th>
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<tr>
<td></td>
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</table>

C

Group 2

% innervated NMJs

Age (days)

0  20  40  60  80  100  120  140  160  180

0  20  40  60  80  100

0  20  40  60  80  100

0  20  40  60  80  100
Figure 4.10 The enzymatic activity of MMP-9 inhibits reinnervation, but is not involved in early pathology that results in the initial FF axonal die-back

A. Experimental timeline. All mice were SOD1G93A. Animals in “Group 1” were treated with SB-3CT (25 mg/kg, i.p.) from P35 to P50, daily except for a 1 day holiday after every 5 days of injections. Animals in “Group 2” were treated with SB-3CT (25 mg/kg, i.p.) from P55 to P75 every day.

B. Results of inhibitor treatment on Group 1. There is no difference in muscle innervation between the groups that received 2 weeks of vehicle treatment (black bar) versus 2 weeks of treatment with SB-3CT (red bar).

C. Results of inhibitor treatment on Group 2. When given daily from P55-P75, surviving animals showed greater amounts of innervation at P75 (blue point) than vehicle-treated controls (yellow point) or than other control SOD1G93A mice at P60. This suggests that there was a greater amount of reinnervation in the inhibitor treated mice.
Figure 4.11

**A**

<table>
<thead>
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**P60 WT, lumbar SC**

**P60 G93A, lumbar SC**

**B**

**C**

**D**

**E**

**F**

**G**

**H**

**I**

**+ no inhibitor**

**+ MMP9-Inhibitor I**

**+ SB-3CT**
Figure 4.11 In situ zymography (ISZ) is an unreliable indicator of MMP-9 activity

A. Table showing the rankings for reliability of signal obtained using various tissue preparation methods. To evaluate these protocols, 2 positive control tissues were tested from mouse (placenta and spleen) and 1 negative control (midbrain from an Mmp9<sup>−/−</sup> mouse). No tissue preparation was ranked 4 (best), because all samples had at least some fluorescence in the negative control. The method that was chosen for further investigation is marked with an *.

B-C. Representative sections of P60 spinal cord from a WT (B) or G93A (C) mouse prepared for ISZ. ISZ is designed to confirm net functional activity of MMP-9 and is predicted to be in only a subset of cells that are positive for MMP-9 by IHC, which cannot discriminate between active and inactive forms of an enzyme (Vandooren et al., 2013a). However, both of these sections had signal from ISZ beyond what is detected by IHC.

D-I. Sections of TA from either a WT or G93A mouse prepared for ISZ. All sections appeared to give a signal along the length of the muscle fibers, though there was a large amount of section to section variability within a given sample. Pre-treating the muscle sections on the slide with MMP-9 Inhibitor I (E, H), or SB-3CT (F, I), did not quench fluorescence. The baseline level of fluorescence in all samples and lack of inhibition suggested that future use of this assay may be limited.
Chapter 5: Preliminary experiments with other candidate targets

Introduction

Despite decades of research, there is still currently no cure for the devastating effects of ALS. The standard treatment approach for patients is mostly restricted to supportive care, including respiratory and nutritional management. In fact, the only disease-modifying drug approved for ALS by the US Food and Drug Administration (FDA) is riluzole, a drug with multiple potential modes of action, including inhibition of Na⁺ current and glutamatergic transmission (Bellingham, 2011), which provides only modest benefit to ALS patients (Andrews, 2009; Bensimon et al., 1994). Further, though several small molecule chemicals aimed at reducing excitotoxicity, oxidative stress, or inhibiting various apoptotic effectors had were considered promising in preclinical studies (Turner and Talbot, 2008), most have been too toxic or have failed to show significant benefit to patients in clinical trials (Andrews, 2009).

Therefore, more candidate therapeutic targets are desperately needed. Our results with MMP-9 presented in Chapters 2-4 suggest that it is an extremely promising therapeutic target. However, mutant SOD1 mice lacking MMP-9 do eventually exhibit motor neuron degeneration and succumb to the disease, indicating that other mechanisms also contribute to degeneration. Therefore, an ideal treatment strategy may be to intervene in multiple pathways to modify the disease.

In order to identify additional targets, the candidate ALS resistance and susceptibility genes from the microarray were again examined. Of the 18 genes that were co-regulated between the resistant and vulnerable populations, we selected one putative ALS resistance gene,
semaphorin 3e (Sema3e), and one additional ALS susceptibility gene, prolyl hydroxylase 3 (Phd3) for further study.

First, Sema3e encodes a class 3 semaphorin, which are secreted signaling molecules implicated in axonal guidance. Previous reports have shown that, depending on its interactions with receptors, Sema3e can function as either a chemoattractant or chemorepellent during formation of specific neuronal circuits. Specifically, Sema3e induces repellant responses through direct interactions with the PlexinD1 receptor (Plxnd1) (Chauvet et al., 2007; Gu et al., 2005a; Oh and Gu, 2013) or attractant responses through interactions with a PlexinD1-Neuropilin-1 (Nrp1) complex (Chauvet et al., 2007), leading the authors to propose that neuropilins have a gating function in semaphorin-plexin signaling. Further, though both Sema3e and its receptors are abundantly expressed in various brain regions (Watakabe et al., 2006), embryonic Sema3e is restricted to several cervical and lumbar motor pools (Cohen et al., 2005; Livet et al., 2002). Moreover, in mice mutant for Pea3, Sema3e expression is lost and the pattern of monosynaptic sensory-motor connections is altered, further suggesting that it may be involved in the formation of pool-specific circuitry (Livet et al., 2002; Vrieseling and Arber, 2006). This has indeed been shown in the case of the Sema3e+ cutaneus maximus (CM) motor pool, which is characterized by the lack of monosynaptic connections with Ia proprioceptive afferents. Using homozygous null Sema3e<sup>plz/hlz</sup> mice and conditional PlexinD1<sup>flox</sup> mice in which PlexinD1 function in DRG neurons was eliminated by intercrossing with Wnt1<sup>Cre</sup> mice, Pecho-Vrieseling et al. (2009) found that specific expression of Sema3e in the CM motor pool is required to prevent the formation of monosynaptic contacts between these motor neurons and PlexinD1+ Ia proprioceptive afferents. Therefore, this study confirmed a functional role of pool-
specific *Sema3e* expression. However, it still remains to be determined whether *Sema3e* has similar functions in other motor pools, especially in the oculomotor and Onuf’s pools.

Previous work in the Henderson Lab showed that at P7 by ISH, *Sema3e* expression was undetectable in motor neurons of disease-susceptible trigeminal and L5 motor neurons. In contrast, high levels of *Sema3e* were present in the majority of motor neurons of the oculomotor nucleus. Likewise, DL nucleus motor neurons expressed high levels of this transcript. This confirmed the microarray results, which found that *Sema3e* was expressed at ~58-fold higher levels in III and ~69-fold in DL, as compared to L5. These expression patterns suggest that *Sema3e* expression could contribute to neuroprotection. Therefore, we hypothesized that removing this candidate resistance gene from SOD1<sup>G93A</sup> mice should exacerbate the disease and might shorten lifespan or induce muscle denervation in normally resistant pools.

Compared to *Sema3e*, *Phd3* had the inverse expression pattern in the microarray and was highly expressed in subsets of lumbar MNs, but strikingly absent from oculomotor and Onuf’s MNs. *Phd3* is also called *Egln3* and *SM-20* (in rat), and belongs to a family of three vertebrate genes that are homologous to the *C. elegans* gene, egl-9, which is a gene required for normal egg laying behavior and resistance to bacterial pathogens (Darby et al., 1999). This gene encodes a protein that is a prolyl hydroxylase that under normal oxygen tension marks hypoxia-inducible factor (HIF) for proteasomal degradation (Fong and Takeda, 2008). Under hypoxia, PHD3 cannot function and therefore allows HIF to accumulate and activate the transcription of many genes required for adaptation to a low-oxygen environment.

Low PHD3 levels and high HIF levels have been reported in cancers and may be permissive for cell growth within the hypoxic environment of tumors (Yan et al., 2009). In contrast, PHD3 is up-regulated by vascular tissue injury, ageing of cells (cardiac cells), and when
neuronal cells are deprived of neurotrophic support (Fong and Takeda, 2008). Forced PHD3 expression has also been shown to induce the formation of protein aggregates in an oxygen- and hydroxylase activity-dependent manner and these PHD3-induced aggregates have apoptosis-inducing potential in HeLa cells (Rantanen et al., 2008). PHD3 also causes caspase-dependent cell death when microinjected into sympathetic neurons (Lipscomb et al., 2001). Correspondingly, *Phd3* knock-out mice have modestly increased numbers of neurons in the superior cervical ganglion (Bishop et al., 2008). However, the mechanisms by which this occurs, and the degree to which the phenotype is cell-autonomous, were not determined. Moreover, the role of PHD3 in motor neuron death has never been studied.

Though PHD3 has not been directly implicated in ALS, deregulation of oxygen sensing has been (Moreau et al., 2011; Quaegebeur and Carmeliet, 2010). All three prolyl hydroxylases destabilize HIF-1α under normoxic conditions, and thus prevent the cellular responses to hypoxia including activation of transcription of multiple genes such as vascular endothelial growth factor (VEGF) and angiogenin (ANG) (Jaakkola and Rantanen, 2013; Pugh and Ratcliffe, 2003). VEGF has been shown to be neuroprotective in ALS: it delayed disease onset and progression in mSOD1 mice following either genetic overexpression of VEGF (Wang et al., 2007), intracerebroventricular administration of VEGF (Storkebaum et al., 2005), or intramuscular delivery of VEGF-expressing lentiviral vectors (Azzouz et al., 2004). Given that expression of VEGF is blocked by PHD3 (via destabilization of HIF), PHD3 has been implicated in cell death in other cell types, and it is highly expressed in vulnerable motor neurons, but absent from resistant ones, we hypothesized that PHD3 may play a role in motor neuron degeneration in ALS. As such we predicted that *SOD1^{G93A},Phd3^−/−* mice should exhibit a delay in the disease phenotype.
Therefore, I performed the following preliminary experiments to test the potential interest of these two additional candidate therapeutic targets for ALS.

**Results**

**Deleting *Sema3e* does not affect the disease phenotype of SOD1\(^{G93A}\) mice**

Given that *Sema3e* was enriched in oculomotor and Onuf’s nuclei but absent from susceptible L5 motor neurons of WT P7 mice, it was considered a potential resistance gene. As such, we hypothesized that deleting it in SOD1\(^{G93A}\) mice would exacerbate the ALS phenotype. To test this hypothesis, Artem Kaplan crossed homozygous null *Sema3e*\(^{nlz/nlz}\) mice on a mixed background to SOD1\(^{G93A}\) mice on a pure C57BL/6 background. The resulting SOD1\(^{G93A};Sema3e^{nlz/nlz}\) mice had an average lifespan of 140.5±11.6 days, (mean ± S.D., n=8), which was not significantly different from that of the control SOD1\(^{G93A};Sema3e^{nlz/+}\) mice (148.5 ± 27 days, n=3). These values are lower than the average lifespans we found in the SOD1\(^{G93A}\) mice on a pure C57BL/6 background, and this different genetic background might serve to conceal or diminish a potential phenotype. However, Artem did note that the clinical course of these mice was not different from that observed in our C57BL/6 SOD1\(^{G93A}\) colony, with mice developing progressive loss of strength and paralysis with similar kinetics. As an additional check for differences, I then examined the oculomotor nucleus and extrocular muscle innervation in these animals (Figure 5.1). There was very little cell death in the oculomotor nucleus in SOD1\(^{G93A};Sema3e^{nlz/nlz}\) mice and the average number of Chat\(^{+}\) MNs was the same as in the oculomotor nucleus of SOD1\(^{G93A};Sema3e^{+/+}\) controls (average MNs per section: 33.0 ± 1.9 vs. 30.2 ± 3.8; p=0.74). Moreover, the extraocular muscles innervated by this motor pool, *superior rectus* (SR), *inferior rectus* (IR), *medial rectus* (MR), and *levator palpebrae* (LP), were assessed
by immunostaining as in Figure 3.1 and were found to be almost completely resistant to
denervation, even without Sema3e (Figure 5.1D). Therefore, these preliminary data suggest that
Sema3e is not the sole determinant of disease resistance in the oculomotor motor neurons.

**Selective PHD3 expression in motor neurons decreases with age**

Next, the putative susceptibility gene Phd3 was analyzed. The microarray analysis revealed
that Phd3 had the inverse expression pattern of Sema3e, with a high level of expression in
susceptible L5 motor neurons, whereas it was absent from oculomotor and Onuf’s nuclei (fold
changes: DL vs. L5: -13.2, and III vs. L5: -11.9). This selective expression was confirmed by
ISH at P9 in WT midbrain and P7 in spinal MN populations (Figure 5.2). Specifically, Chat was
used to locate the EOM-innervating oculomotor (Figure 5.2A) and trochlear (Figure 5.2C)
nuclei. Directly adjacent sections lacked Phd3 signal (Figure 5.2B, D). In contrast, vulnerable
spinal motor neurons at L5 and L6 had robust Phd3 expression (Figure 5.2F, H). Comparing
Chat and Phd3 expression on adjacent L5 sections suggests that Phd3 is present in nearly all
motor neurons and possibly other cells types as well (Figure 5.2E, F). In L6 cryosections, the
number of Phd3 cells and intensity of the signal within them were lower than Chat in both RDL
and DL, but very high in DM (Figure 5.2G, I).

Because PHD3 is one of three prolyl hydroxylases, the expression levels of the two other
genes on the original microarray were compared (Table 5.1). PHD1 (Egln2) and PHD2 (Egln1)
are expressed at comparable levels to PHD3 (Egln3) at L5 where all three are present at high
levels. However, PHD1 and PHD2 do not show the same selective expression and are also
expressed at very high levels in oculomotor and DL nuclei, where PHD3 is considered absent.
These values remain to be confirmed by ISH.
We next looked at Phd3 expression in lumbar cryosections of adult WT mice and found that Phd3 was only weakly expressed at P30 and P40, compared to P7 (Figure 5.3A-C). By P57, Phd3 was absent from the lumbar spinal cord and similar observations were made at P162 (Figure 5.2 D-E). Therefore, Phd3 expression down-regulates in adulthood in WT mice. This finding is consistent with data generated by Timothy Spencer in the Henderson lab. He analyzed the molecular characteristics of motor neurons retrogradely-labeled from the TA from WT mice at P4, P12, and P60 that were isolated by laser capture microdissection. Using microarray analysis he found that Phd3 expression also decreases over time in an identified fast motor pool (Figure 5.3F).

**Genetically ablating Phd3 does not affect motor neuron death during development or disease**

Despite the down-regulation of Phd3 in susceptible motor pools in the adult mouse, we wanted to investigate if genetically deleting Phd3 protected SOD1<sup>G93A</sup> mice, as with MMP-9. First, because it was reported that Phd3 knock-out mice have modestly increased numbers of neurons in the superior cervical ganglion (Bishop et al., 2008), we established whether there was a role for PHD3 in developmental motor neuron death by counting MNs in L5 and L6 of WT and PHD3 KO mice. MN survival was not significantly increased in the Phd3<sup>−/−</sup> mice compared to WT controls at P30 in any of the 3 motor pools studied: L5 (21 ± 1.2 vs. 18.8 ± 0.8, p=0.18), RDL (10.7 ± 0.9 vs. 9.3 ± 0.8, p=0.32), and DL (7.87 ± 0.4 vs. 6.8 ± 0.3, p=0.14), though increasing the sample size here would strengthen this claim (Figure 5.4).
Next we crossed SOD1\textsuperscript{G93A} mice in our C57BL/6 colony to the \textit{Phd3}\textsuperscript{+/−} mice on a CD-1 background and compared the average lifespan of the SOD1\textsuperscript{G93A} littermates with or without PHD3. The SOD1\textsuperscript{G93A} mice now on a mixed background had a statistically shorter lifespan than the C57BL/6 SOD1\textsuperscript{G93A} mice in our colony, with a 7 day reduction in median survival time (log-rank test= 4.2, p=0.04, black lines in Figure 3.8 and 5.5). Next, we compared littermates on the mixed background with one or both alleles of \textit{Phd3} deleted. There was no significant difference in the median lifespan of these mice (log-rank test= 5.3, p=0.07, Figure 5.5). Because the breeding of these animals was slower than expected and the number of \textit{Phd3}\textsuperscript{−/−} live births among the offspring of heterozygous matings was less than expected, in agreement with an earlier study (Bishop et al., 2008), only 19 animals total were included in this study. It is possible that a larger sample size may have resulted, contrary to expectations, in a significant exacerbation of the disease phenotype, since there was a trend that SOD1\textsuperscript{G93A} mice with reduced or absent PHD3 levels die earlier (median survival for SOD1\textsuperscript{G93A};\textit{Phd3}\textsuperscript{+/+}, and \textit{Phd3}\textsuperscript{+/−}; \textit{Phd3}\textsuperscript{−/−}: 150, 138, and 135 days, respectively). Overall, we no longer consider PHD3 a promising therapeutic target for ALS, as \textit{Phd3} is not expressed in adult motor neurons and genetically deleting it does not protect mSOD1 mice.

**Discussion**

In this chapter, we present the results of experiments to explore a potential role for two additional candidate genes in the selective resistance or vulnerability of different motor pools in ALS. In a first set of experiments, we genetically removed a candidate resistance gene, \textit{Sema3e}, from mutant SOD1 mice, and in the second set we removed another candidate susceptibility gene, \textit{Phd3}. Neither genetic manipulation had a significant effect on the disease progression, as
measured by lifespan or changes in muscle innervation. These results show the importance of careful functional validation of the candidate genes from the initial screen.

Differential enrichment of *Sema3e* in the oculomotor and DL motor neurons raised the possibility that this gene could be involved in the resistance of these motor neurons to degeneration in mutant SOD1 mice. However, functional studies crossing *Sema3e*\(^{nlz/nlz}\) and SOD1\(^{G93A}\) mice showed that loss of *Sema3e* function did not appear to affect the resistance of the oculomotor nucleus, as it did not affect average motor neuron numbers or the amount of innervation of the extraocular muscle. Further, Artem Kaplan’s earlier work did not show a significant effect on lifespan, though increasing the sample size and also comparing the SOD1\(^{G93A};Sema3e^{nlz/nlz}\) mice to a SOD1\(^{G93A};Sema3e^{+/+}\) group would further strengthen this analysis. Therefore, these results argue against a prominent role of *Sema3e* in disease resistance.

When the analysis was limited to genes whose expression levels showed >10-fold differences, 14 genes were more strongly expressed in vulnerable motor neurons than in the two resistant populations. To determine which of these 14 genes to further investigate, we considered a number of selection criteria, as discussed in Chapter 1. For example, when we chose *Mmp9* as a target, we did so because its expression was motor neuron-specific, it was expressed selectively in motor neurons throughout adulthood, and its ranking was low in both individual pairwise comparisons (III vs. L5) and (DL vs. L5), but high using the three-way triangulation, suggesting that it had a greater chance of being linked to ALS rather than to specific properties of either pool. Further there were numerous reagents available to study MMP-9 and there was a preexisting literature indicating a potential role in ALS. *Phd3* did not meet all of these criteria. Though we did confirm by ISH that *Phd3* was absent from EOM-innervating pools and expressed at low levels in DL, it appeared that *Phd3* was also expressed in
other cell types besides motor neurons at lumbar spinal cord levels at P7. Further, Phd3 was not stably expressed into adulthood, as we found variable expression at P30 and P40, and none at P57 or P162. It remains a possibility that this expression profile changes in a disease context given that PHD3 has a role in oxygen sensing and oxidative stress likely plays an important role in ALS pathogenesis (Barber and Shaw, 2010). Therefore, it may still be useful to map the expression of Phd3 in SOD1G93A motor pools at disease-relevant time-points. Further, the value added by triangulation rank for Phd3 was only moderate (Table 1.1), with a “value added” score of 47 (compared to 91 for Mmp9).

However, PHD3 had been implicated previously in cell death and knock-out mice were available to us, so we performed the cross with SOD1G93A mice as a preliminary study to assess a potential neuroprotective role for prolyl hydroxylase inhibition. Unfortunately, genetically removing one or both alleles of Phd3 did not prolong lifespan, and may have even diminished overall survival, though the number of mice included in this study was too low to detect a significant change. It may be worthwhile to examine the effect of Phd3 removal at an earlier disease stage, as it is possible that early protective effects exist, but are masked at late disease stages because the Phd3+/− have non-motor phenotypes that contribute to premature death. We did not characterize age-matched Phd3+/− without mutant SOD1, so this remains an open question. It has been reported that Phd3 null mice display decreased apoptosis in SCG neurons, reduced adrenal medullary secretory capacity, abnormal adrenal medulla morphology, reduced circulating adrenaline and noradrenaline levels, and reduced systolic blood pressure (Bishop et al., 2008; Chen et al., 2012). No reports suggest that these mice have reduced survival, but the phenotyping studies were performed on animals between 2 months and 6 months of age, so differences still may emerge at later time points.
Finally, follow-up studies are needed to examine the possibility that other PHD isoforms compensate for the Phd3 deletion. PHD2 and PHD3 are suggested to have partially redundant roles \textit{in vivo} with combined loss resulting in increased HIF1α levels as well as increased HIF target gene expression (Minamishima et al., 2009). Further, loss of Phd2 results in a small upregulation in Phd3 levels (Takeda et al., 2006). To my knowledge, whether the reverse is true has not yet been investigated. However, the results of the microarray suggest that the other PHD isoforms are expressed at similar levels at L5, but 7-12 times higher in the resistant motor pools (Table 5.1). Therefore, there is a strong possibility that if PHD3 is related to motor neuron degeneration at all, its role could be redundant. This is a significant disadvantage for any candidate therapeutic target.

Overall, therefore, determining the precise contribution of to Phd3 and Sema3e to disease vulnerability or resistance, if any, awaits multiple future experiments. For example, virally introducing Sema3e to vulnerable pools, such as TA-innervating MNs, and looking for protective effects may be more clinically meaningful than genetically deleting Sema3e and investigating changes in EOM-innervation. However, at this point neither target is considered a high priority for further work to develop viable therapeutic strategies for ALS. However, there is evidence that extraocular muscle and pelvic sphincter-innervating motor pools are also preserved in spinal muscular atrophy (SMA) patients and mouse models (Lee et al., in preparation). It is possible that these specific genes may play a more prominent role in that disease. Ongoing experiments in collaboration with Justin Lee to perform crosses of Phd3 knockout mice with SMA-Δ7 mice will answer this question.

New candidate genes are still needed for the treatment of ALS and these targets may still be revealed by further analysis of the original microarray followed by functional validation.
studies in vivo. In the future it may also be beneficial to add more motor pools with different degrees of resistance to the gene list already identified from the extreme ends of the resistance and vulnerability spectrum (that is, the most resistant EOM and sphincter-innervating MNs and the most vulnerable lumbar motor neurons). This even finer grained analysis could potentially reduce the number of potential targets, allowing us to more easily identify genes that co-segregate best with resistance or vulnerability.
Figure 5.1

Oculomotor Nucleus

SOD1^{G93A};Sema3e^{+/+}  SOD1^{G93A};Sema3e^{-/-}

A  B

C

Avg # MNs

Semae^{+/+}  Semae^{+-/-}

D

% Innervated NMJs

SR  IR  MR  LP

SOD1^{G93A}, terminal
Figure 5.1 Ablation of *Sema3e* in SOD1\textsuperscript{G93A} mice does not confer vulnerability onto the EOM motor pool

A – B. Representative cryosections of the oculomotor nucleus stained with ChAT (green) at end-stage from a SOD1\textsuperscript{G93A} control animal (A) or a SOD1\textsuperscript{G93A};*Sema3e*\textsuperscript{−/−} mouse.

C. There is no difference between the average number of motor neurons in the oculomotor nuclei of SOD1\textsuperscript{G93A};*Sema3e*\textsuperscript{+/+} mice (black bar) and SOD1\textsuperscript{G93A};*Sema3e*\textsuperscript{−/−} mice (red bar) at end-stage, showing that ablation of *Sema3e* did not lead to increased cell death in this motor pool (n=3, mean ± SEM).

D. There are also no differences between the degree of innervation of extraocular muscles innervated by the MNs of the oculomotor nucleus (SR, IR, MR, and LP) in SOD1\textsuperscript{G93A};*Sema3e*\textsuperscript{+/+} mice (black bars) and SOD1\textsuperscript{G93A};*Sema3e*\textsuperscript{−/−} mice (red bars) at end-stage (n=3, mean ± SEM). None of the muscles showed significant loss of NMJ innervation in either group, relative to WT controls (not shown).
Figure 5.2

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| H         |      |
**Figure 5.2** *Prolyl hydroxylase 3 (Phd3)* is expressed in subsets of spinal motor neurons but is absent from oculomotor and DL nuclei

A-D. ISH on adjacent cryosections from P7 and P9 WT mice reveal that *Phd3* is absent from the oculomotor (B) and trochlear (D) nuclei, detected by their *Chat* expression (A,C).

E-H. Both *Chat* and *Phd3* are expressed in wild type spinal cord at P7. *Phd3* appears to be present in both motor neurons and other cells in L5 (compare E and F). In L6, the levels of *Phd3* are lower, especially in DL, but very high in DM (H).
Table 5.1 Gene expression of other PHD genes

Averaged gene expression values obtained from the microarray expressed and fold change over the averaged values for *Egln3* (red text) in the 3 motor pools sampled. The other PHD isoforms, PHD1 (*Egln2*) and PHD2 (*Egln1*), are expressed at comparable levels to PHD3 (*Egln3*) at L5 where all three are present at high levels. However, PHD1 (*Egln2*) and PHD2 (*Egln1*) are expressed at high levels in oculomotor and DL nuclei, where PHD3 is considered absent.

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Table 5.1 Gene expression of other PHD genes
Figure 5.3

F

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Figure 5.3 Motor neuron expression of Phd3 decreases with age in WT mouse lumbar spinal cord

A – E. Phd3 is expressed at high levels in the ventral horn of a cryosection of lumbar spinal cord from a P7 wild-type mouse (A), is expressed at much lower levels at P30 (B) and P40 (C), and is absent from lumbar spinal cord sections at P57 (D) and P162 (E).

F. Raw expression levels of Phd3 in laser-captured TA MNs from WT mice at P4 (blue bar), P12 (red bar), and P60 (green bar) assessed by microarray shows a decrease in Phd3 expression values with age. Data from T. Spencer
Figure 5.4

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A

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Avg # MNs

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Figure 5.4 Genetic ablation of Phd3 does not result in reduced susceptibility of motor neurons to developmental cell death

A-B. Immunostaining for ChAT (green) in P30 spinal cord of Phd3−/− mice at lumbar level L5 (A) and L6 (B).

C. Deleting Phd3 has no effect on MN numbers at P30 in L5 or L6 as there are no significant differences between numbers of ChAT+ cells in Phd3−/− (grey bars) and WT (black bars) mice (n=3, mean ± SEM).
Figure 5.5 Lifespan is not significantly altered following deletion of one or both alleles of Phd3 in SOD1\textsuperscript{G93A} mice.

Kaplan–Meier plot showing the cumulative probability of survival of SOD1\textsuperscript{G93A};Phd3\textsuperscript{+/-} (red), SOD1\textsuperscript{G93A};Phd3\textsuperscript{+-} (blue), and SOD1\textsuperscript{G93A};Phd3\textsuperscript{++} mice (black) (n=5-7 animals per genotype; log-rank test= 5.3, p=0.07).
Chapter 6: General Discussion

All neurodegenerative diseases are characterized by dysfunction and death of select neuronal populations. The term “selective regional vulnerability” was coined almost 80 years ago by Alfred Meyer, who declared that “the most fascinating fundamental question is the problem of selective vulnerability in the brain” (1936). He described selective lesions in epidemic encephalitis, Pick’s disease, and Wilson’s disease. Since this seminal paper, it has been widely recognized that nearby populations of neurons with generally similar characteristics and functions can have greatly divergent responses to disease-induced cell damage in neurodegenerative diseases. For example, in AD there is differential damage to pyramidal neurons in entorhinal and hippocampal subregions, as well as different cerebral cortical layers (Hyman et al., 1984). Specifically, in the hippocampus, CA1 pyramidal neurons are readily injured or die under conditions that for the most part do not affect the adjoining CA3 pyramidal neurons or dentate granule cells (Michaelis, 2012). In Parkinson’s disease, dopaminergic mesostriatal neurons are susceptible to degeneration while mesolimbic and mesocortical dopaminergic neurons are more resistant. And in ALS certain motor neuron subsets show different degrees of resistance to degeneration, as has been observed in patients and confirmed in the most widely used mouse model (mutant SOD1 mice) by us and others. Importantly, all three of these neurodegenerative diseases are strongly associated with ageing.

In this thesis we have used a precise analysis of the molecular diversity within motor neuron subpopulations to answer broader questions about the timing and regional selectivity of neurodegenerative diseases. Focusing on ALS, we show that the MMP-9 expression is localized
to the vulnerable population of motor neurons and that its expression begins at the age when the first signs of pathology are reported in ALS model mice. Further, we demonstrate that MMP-9 is functionally contributing to the disease. In this discussion, I will first briefly review these findings and put them into context. I will then discuss advantages and also some drawbacks to the approach that we took, as well as highlight some unanswered questions and future directions. Finally, the clinical implications of this thesis will be examined.

**Localization of MMP-9 in disease vulnerable populations**

We found that MMP-9 expression is restricted to the most vulnerable class of neurons (motor neurons), the most vulnerable populations within this class (lumbar motor neurons, but not the resistant oculomotor or Onuf’s nuclei), and the most vulnerable neuronal subtype within a vulnerable region (fast α-motor neurons). This pattern suggests that the tightly restricted expression of MMP-9 in fast motor neurons effectively gates the onset of disease that is caused by the widespread expression of mutant SOD1 in the SOD1<sup>G93A</sup> mouse. Thus, the specific localization of MMP-9 may explain the highly selective degeneration of FF motor neurons on a clear cellular basis. Our identification of MMP-9 as a marker of susceptible populations will make it possible to someday systematically assess potential molecular differences that distinguish all vulnerable motor neurons by assessing gene expression profiles in MMP-9 expressing and MMP-9-negative motor neurons, isolated by laser capture microdissection.

Moreover, though other potential markers of fast and slow motor neurons have been reported previously (Chakkalakal et al., 2010; Enjin et al., 2010; Kanning et al., 2010), no known markers had been identified to distinguish FF and FR motor neurons in rodents. Based on motor
neuron size and backfill data from muscles of known fiber type composition that I presented in Chapter 2, we hypothesize that the population that shows intermediate size and lower levels of MMP-9 could correspond to FR motor neurons, though this awaits further confirmation.

It was serendipitous that our approach looking at genes that were absent from oculomotor and Onuf’s nuclei uncovered a marker for fast motor neurons and this unexpected result may help to inform the mechanism of disease vulnerability. For example, aside from lacking MMP-9, type S motor units have other features in common with extraocular and pelvic motor units such as high activity levels. Specifically, different muscles exhibit significant variations in the cumulative time they are active throughout an 8 hour period, with postural muscles such as Sol active 14% of the time compared to 2% for the TA (Monster et al., 1978). When continuous recordings were made for extended periods from single motor units in the fast extensor digitorum longus and the Sol muscles of freely moving adult rats, it was revealed that Type S motor units indeed fired more frequently, with an active duty that is approximately 300 times greater than Type FF motor units (Hennig and Lømo, 1985; Hennig and Lømo, 1987). Strikingly, extraocular motor units are active almost continuously during the day and during REM sleep (Robinson, 1978) and pelvic sphincter motor units are active throughout the day and at night ceasing only during brief periods of urination and defecation (Enck and Vodusek, 2006). This suggests that levels of daily activity of motor pools are correlated to their disease resistance. A possible explanation may be that highly active motor neurons may have evolved to deal with the high metabolic burden required to maintain sustained nerve conduction and synaptic transmission, as recently suggested in a report by the Caroni group in which the authors showed that increasing activity levels made motor units more ALS resistant (Saxena et al., 2013). Whether MMP-9 could modulate firing properties of fast motor neurons to make them more vulnerable will be an
important question to address in future studies. Thus, MMP-9 is potentially very useful for studies aimed at understanding basic motor neuron biology, in addition to its relationship to disease.

**Pathological changes in mSOD1 mice do not begin until MMP-9 is expressed**

Further, in ALS mice, although mutant SOD1 is expressed embryonically, the first pathological changes *in vivo* are only detected in the first postnatal week (Kanning et al., 2010). Specifically, the earliest change reported is an increase in the electrical excitability of hypoglossal motor neurons between P4 and P10 (van Zundert et al., 2008). The authors also noted behavioral changes (errors in forelimb placement and delays in righting) that can occur as early as P10. Similarly, misfolded SOD1 is detected in a subpopulation of alpha-MNs in the lumbar spinal cord in mutant mice from P7 on, but is not yet there at P1 (Saxena et al., 2013). Strikingly, MMP-9 is not expressed by embryonic motor neurons and is first detected in the spinal cord only around P5. Therefore, the timing and localization of the expression of MMP-9 suggest that it may be gating the disease phenotype.

**Functional involvement of MMP-9 to disease vulnerability**

Using multiple approaches to control MMP-9 expression and activity, we found that MMP-9 plays a major role in motor neuron degeneration in ALS model mice. This action is cell-autonomous in motor neurons and is at least partially mediated through the enzymatic activity of MMP-9 in the spinal cord. Moreover, MMP-9 influences the degenerative process early, as it is needed for full CRT down-regulation and the induction of ER stress to occur. However, MMP-9 is not alone sufficient to trigger neurodegeneration since it is expressed in many wildtype motor
neurons and does not induce axonal dieback when virally introduced into extraocular muscle- or Sol-innervating motor neurons. These findings are significant in that we have shown that we can manipulate the molecular make-up of FF motor neurons postnatally so that they more closely resemble resistant motor neurons. The strong protection thereby conferred provides hope that not only MMP-9 but also other genes with subtype-specific expression may provide a basis for rational therapies in ALS and other neurodegenerative diseases.

Advantages of our approach

The work presented in this thesis has several strengths. First, though over the last few years several groups have taken a similar laser-capture dissection and microarray approach of resistant and vulnerable populations to study potential mechanisms of degeneration in both PD and ALS (Brockington et al., 2013; Chung et al., 2005; Duke et al., 2007; Greene et al., 2005; Hedlund et al., 2010; Lu et al., 2006), we have extended the analysis in a number of ways. First, prior studies did not map out the expression of differentially expressed genes in the same detail as I have in this thesis. For example, a study comparing global gene expression profiles in motor neurons of the oculomotor nucleus to the hypoglossal nucleus found hundreds of differentially expressed genes (Hedlund et al., 2010). Of these, the authors selected insulin-like growth factor II (Igf2) and guanine deaminase (Gda) for further study and confirmed that they are present in oculomotor neurons, but absent from hypoglossal and cervical spinal motor neurons only by unquantified immunostaining of sections of rat tissue, from animals whose age and genotype were not specified in the text. However, Hedlund et al. (2010) did present some functional data for these genes, showing that pretreatment with recombinant IGF-2 and GDA were protective in
vitro against glutamate-induced neurotoxicity. Unfortunately, these experiments were not followed with any experiments to remove these genes and assess susceptibility or in vivo analysis. In contrast, in this thesis I take several complementary approaches to demonstrate in vivo therapeutic relevance of a differentially expressed gene, Mmp9.

Moreover, compared to earlier profiling studies, our work also produced a smaller list of targets because of the triangulation method generated by including an additional resistant pool (Onuf’s nucleus). Examining genes that were co-regulated between oculomotor and Onuf’s nuclei ensured that we were not looking at genes that just controlled rostro-caudal identity or something else peculiar to individual motor demands, but rather co-segregated with resistance. In fact, the triangulation provided by studying these two resistant populations led us to a target that is unlikely to be deduced from simple comparison of oculomotor and vulnerable motor neurons. Specifically, MMP-9 did not rank above #24 on any pairwise comparison, and so we would not have identified MMP-9 if we had looked only at genes that were top in an individual comparison. For instance, MMP-9 was not specifically identified in a very recent study by Brockington et al. (2013), in which they used microarray analysis to compare the gene expression profiles of isolated motor neurons from the oculomotor nucleus and lumbar spinal cord from human post-mortem tissue from four neurologically normal control subjects. They noted that extracellular matrix components, including seven different collagen subunits, were expressed at higher levels in lumbar motor neurons than oculomotor neurons, but they did not specifically pull out MMP-9 as being a gene of interest. However, when we looked at MMP-9 values from these samples as reported in their repository in the online GEO omnibus database, we found that MMP-9 was considered present in lumbar MNs and absent in oculomotor MNs, with levels that are ~2.5-fold lower in oculomotor nucleus than in lumbar motor neurons. Still,
even with this triangulation approach, we found genes that did not modify the disease course in vivo (Sema3e and Phd3), demonstrating that, though the list of identified genes is an enriched source of candidates, the genes revealed by this approach may also not be involved in resistance or vulnerability to degeneration in ALS.

Another advantage to our approach is that rather than just looking at a late stage event, we track the expression and effects of MMP-9 throughout life (by the use of Mmp9 null mice), from postnatal onward (by the use of AAV knock-down and expression), and from after start of denervation (using a pharmacological inhibitor). These experiments have allowed us to identify MMP-9 as an early disease trigger. Further, we have shown that motor neurons are the cellular site of action of MMP-9 at these early disease stages, rather than invading microglia. Moreover, all of this work was done in vivo, which I think is a real strength given that MMP-9 is a secreted enzyme and so is likely affecting other nearby cell-types and pathologically altering a complicated extracellular environment. It would be hard to recapitulate this in a cell culture system. Though undoubtedly it would be more convenient to study MMP-9 in an in vitro system such as from mouse embryo derived- or stem-cell derived- MNs (discussed below), the in vivo TA MN targeting approach that I describe in Chapter 4 can serve the same purpose. In fact, studying the function of human MMP-9 in a mouse otherwise devoid of the enzyme bypasses some of the limitations of in vitro systems, including the loss of functional connectivity and alterations in 3D cytoarchitecture. Moreover, it is not yet possible to culture FF motor neurons specifically and therefore potentially relevant cofactors to MMP-9 could be absent from currently available model systems.

Finally, this study shows the potential for using AAV as an experimental tool to assess gene function in intact animals. This gene targeting approach can produce tissue-specific gene
ablation results similar to those obtained with conditional knock-out mice, in which Cre recombinase, expressed under the control of a brain region-selective promoter, excises DNA sequences located between loxP sites in a spatially controlled manner (Sauer, 1998; Tsien et al., 1996). However, the generation and breeding of conditional knock-out mice is a resource- and time-intensive task. Further, the breeding strategies can increase the variation in the genetic background of the resultant offspring, potentially confounding behavioral and other analyses (Kaspar et al., 2002). Also, depending on the promoter, excision may occur during development, causing disadvantageous side effects. Therefore, our AAV approach in collaboration with the Aebischer lab offers several advantages for spatially controlled gene ablation. First, AAV viruses can be generated, administered, and results can be analyzed over the course of several months versus years for the conditional knock-out approach. Moreover, fewer animals are needed because no intermediate breeding generations are required. Also, AAV-mediated gene knock-down occurs exclusively postnatally. Finally, in some instances such as with intramuscular injections of AAV6, pools within a single animal can be compared, decreasing the variability and further strengthening the conclusions. Therefore, we believe that increased preclinical use of AAV vectors will be valuable for the future evaluation of candidate therapeutic targets for ALS and in other disease model mice.

**Drawbacks to our approach**

Though there are many advantages to the experimental strategies that we have taken, there are a few drawbacks to our experiments that are also worth noting. First, a limitation of the microarray approach is that we look at intrinsic differences between motor neurons, but do not
examine disease-responsive changes in gene expression that may at least partially underlie disease resistance. Further, we are unable to detect post-transcriptional (e.g. splicing) or post-translational (e.g. phosphorylation) differences by our profiling and comparisons. Finally, by our triangulation approach, there could be real oculomotor-specific or Onuf’s-specific resistance genes excluded.

Another disadvantage to the experimental strategy that we have taken in this thesis is that even though AAV knock-down or overexpression is less time intensive than comparable genetic approaches, there still involves waiting at least 6 weeks between viral administration and analysis. Thus, biochemical experiments to better understand potential molecular pathways in which MMP-9 acts would be aided by having an in vitro model system. Beyond their convenience, the primary advantage of in vitro studies is that they offer a controlled environment to test specific cellular and molecular hypotheses relatively quickly. Unfortunately, MMP-9 is not expressed in ES-MNs, which is perhaps expected given that we show in Chapter 2 that MMP-9 is not expressed in motor neurons in vivo until about P5. Further, though motor neurons generated from mouse embryonic fibroblasts (iMNs) do tend to express higher levels of MMP-9 than stem cell-derived MNs, the expression level is still rather minimal (personal communication, Eggan Lab). However, the same fibroblasts grown for 15 days in serum-free media have high levels of MMP-9 and the members of the Eggan lab have noticed that several pathways expressed in fibroblasts are maintained in iMNs made from them. It is therefore possible that iMNs made from fibroblasts grown for 15 days in serum-free media would have high levels of MMP-9. It is also possible, but as of yet untested, that iMNs from adult fibroblasts would produce MNs with MMP-9 expression.
Unanswered questions and future directions

Although I argue above that these studies have advanced the field significantly, they open up as many questions as they answer and there are many future research directions that warrant more study. For example, what if anything about MMP-9 itself makes motor pools “fast”? What other subtype specific elements in addition to mutant SOD1 and MMP-9 lead to degeneration? How can we better understand the relative roles of the enzymatic vs. non-enzymatic activities of MMP-9 in driving neurodegeneration? What are the pathways downstream of MMP-9, and how do these interact with mutant SOD1 and, potentially, other disease triggers? And, how can we apply what we learn about the mode of action of MMP-9 to designing therapeutic strategies for ALS patients? These are only some of many questions that arise from this work, and, though I will discuss each in more detail below, this is not a complete list.

Does MMP-9 make motor units fast?

Skeletal muscle is highly plastic and is able to switch fiber type (and thus alter its total composition of fiber types), in order to meet particular physiological demands or in response to injury by damaging agents or disease (LeBrasseur et al., 2011). Several factors can induce fiber type switching, with the best-studied factor being changes in nerve activity or loading (Schiaffino and Reggiani, 2011). The role of the nerve in affecting the fast/slow properties was first demonstrated in the cat, where slow muscles became faster when reinnervated by a fast nerve, and fast muscles become slower when reinnervated by a slow nerve (Buller et al., 1960). Subsequent studies using different impulse patterns to reproduce the firing pattern of fast and slow motor neurons in innervated or denervated muscles showed that the effect of the nerve is
mediated by these specific impulse patterns (Pette and Vrbová, 1999; Schiaffino and Reggiani, 2011; Vrbová and Ward, 1981).

Our examination of the superficial versus deep layers of the TA as well as our comparison of the TA and the control slow Sol muscles suggest that fast SOD1<sup>G93A</sup> motor neurons without MMP-9 adopt the axonal die-back properties of slow motor neurons. Strikingly, this is not the first time MMP-9 has been reported to play a role in regulating fast/slow motor properties. In fact, Mehan et al. (2011) showed that muscle fiber cross-sectional area (CSA) and fiber type distribution were significantly altered in female MMP-9 null mice compared to female WT mice. Specifically, the absence of MMP-9 significantly decreased the CSA of different types of skeletal muscle fibers expressed in the TA, GC and Sol muscles, including slow fibers. Consistent with what we would predict, this effect was most dramatic in the fastest fibers (MyHC type IIb-expressing fibers) in TA and GC. However, in contrast to our expectations, they also report that the percentage of MyHC IIb-expressing fibers was greater in both the TA and GC of MMP-9 null mice, though this was only measured by staining for different MyHC isoforms and not by examining functional changes. The authors also note that these changes appear to occur in the absence of significant changes in type IV collagen staining and are not accompanied by compensatory changes in MMP-2 expression in the muscle. They further show that neither MMP-9 inactivation nor the compensatory fiber type or size changes accompanying it resulted in a change in hindlimb twitch force output in an in vivo functional test (HEFT). This result is consistent with ours, in that we detected no motor phenotype in the Mmp9<sup>−/−</sup> group in either the accelerating rotarod task or in a swim test. Thus, though this particular study is consistent with our data and predictions for Mmp9<sup>−/−</sup> muscle with regard to their reports of unchanged contractile force in hindlimb muscles and reductions in fast fiber CSA, the direction
of fiber-type composition alteration from slow to fast does not align simply with our axonal die-back data.

However, a subsequent study reported that mice engineered to overexpress active MMP-9 predominantly in skeletal muscle using the MCK promoter had an increased proportion of fast-type glycolytic fibers (Dahiya et al., 2011). When the authors focused on the Sol muscle, they found that type IIB and type IIx fibers, which were not detectable in the WT Sol muscle, emerged in the MMP-9-overexpressing Sol muscle. Interestingly, there was no significant difference in fiber-type composition in the TA, likely because the TA muscle is already predominantly fast. If we compare these results to the data we generated, they are in general conceptual agreement. However, we did not see an increase in muscle denervation when MMP-9 was virally introduced into the Sol motor pool (Figure 4.6). The difference here could result from the fact that the MMP-9 we added was full-length and needed to be activated, in contrast to the enzymatically active form that Dahiya et al. expressed (2011). Another possible explanation may be that the cellular source of MMP-9 that results in fiber type switching is not neuronal. In fact, a wide range of cell types found within skeletal muscle can express MMP-9 during post-damage repair including endothelial cells (Kherif et al., 1998), satellite cells (Chen and Li, 2009; Fukushima et al., 2007; Guérin and Holland, 1995), and inflammatory cells (Choi and Dalakas, 2000; Kherif et al., 1999). Our knockout studies do not distinguish between the possible cellular sources of the MMP-9 that leads to the fast to slow switching. In the future it will be interesting to examine the denervation of the skin facing versus bone facing TA compartments in 1) mutant SOD1 mice with MMP-9 silenced only in motor neurons at later time-points (for instance, with an i.c.v. injection of AAV6.shMMP9) and 2) in SOD1<sup>G93A</sup>;<i>Mmp9</i><sup>−/−</sup> mice with MMP-9 overexpressed only in motor neurons. We could also perform the converse experiments by
delivering cDNA or shRNA packaged in a different serotype (AAV7) into the TA, because that serotype will very efficiently transduce muscle, but is not retrogradely transported to MNs (Gao et al., 2002).

Therefore, these results and ours suggest that MMP-9 critically affects the functional properties of motor neurons and the muscle fibers they innervate. How could MMP-9 alter the properties it affects and regulate this plasticity and adaptation? From the neuronal end, given that muscle plasticity reflects motor neuron plasticity, MMP-9 may exert its effect by altering neuronal activity as it has been widely reported to do in the hippocampus (Gorkiewicz et al., 2010; Michaluk et al., 2011; Vandooren et al., 2013b; Wilczynski et al., 2008). It will be interesting to measure the excitability of TA motor neurons with or without MMP-9. Based on recent results that showed that increased excitation of motor neurons can prevent their degeneration (Saxena et al., 2013), we would predict that expressing MMP-9 somehow decreases motor neuron excitability. However, this prediction runs counter to results in the hippocampus, wherein it was demonstrated that MMPs increase neuronal excitability and burst dynamics, as measured by increased overall spike counts, burst number, and burst duration (Niedringhaus et al., 2012).

On the muscle side, MMP-9 from fast motor neurons may be released and activated as needed at the NMJ. Once activated, MMP-9 mediated remodeling of the extracellular matrix (ECM) could allow muscle fibers to adapt their size or thickness, their internal structure and their extracellular surroundings (for example, capillary density) to better cope with their metabolic activities (Schiaffino and Reggiani, 2011). For example, one (possibly simplistic) scenario is that MMP-9 in fast fibers degrades ECM proteins, thus causing a laxation of the restriction by
ECM on muscle fiber, allowing for increased fiber growth. In this scenario, when MMP-9 is removed, unchecked ECM proteins could create a physical barrier that restricts fiber size.

Furthermore, degradation products of ECM components can be more than just debris; several fragments of structural proteins have biological activity after proteolytic processing, and MMP activity can therefore release growth factors stored in the ECM to induce these changes (Rullman et al., 2009). Follow-up experiments to measure gelatinolytic activity in muscle and/or levels of various ECM components in fast fibers with or without MMP-9 in the motor neurons that innervate them are potentially interesting and feasible experiments. Further, it would be relatively straightforward to look at time-points in between P50 and P100 in the SOD1<sup>G93A</sup>;<i>Mmp9</i><sup>−/−</sup> muscles assess whether there are still distinct waves of denervation (albeit, delayed relative to SOD1<sup>G93A</sup>;<i>Mmp9</i><sup>+/−</sup> littermates), or if all of the denervation is compressed into a single wave, which would be consistent with a switch from FF to FR in the absence of MMP-9. Therefore, studying the role of MMP-9 in shaping motor units’ fast properties is an exciting avenue for future research.

Unidentified elements that are specific to fast motor neurons in the mSOD1/MMP-9 pathway lead to degeneration

Another exciting open question arises from the observation that the activation of the pathway for axonal die-back requires neuronal subtype-specific elements in addition to MMP-9 and SOD1. Specifically, we showed in Chapter 4 that MMP-9 induces degeneration in fast, but not slow, motor neurons of ALS mice. What are the additional molecular player(s) that are present in fast but not slow pools that help to mediate the effect of MMP-9? The microarray
reported by Saxena et al. (2009) comparing fast (GC-L1 -innervating) and slow (Sol innervating) motor neurons may be a valuable resource to find other elements that are expressed in these vulnerable lumbar MNs that could be contributing to the pathology in these cells.

Based on our data with the pharmacological inhibitors of MMP-9, we know that enzymatic activity is at least partially involved. As such, we expect that we will find a factor (or factors) to be either upstream of MMP-9 and thus activate it from its proform, or substrates of MMP-9, whose cleavage has negative consequences. An example of a potential substrate that could lead to toxicity is stromal cell–derived factor 1α (SDF-1), a chemokine overexpressed during HIV infection that has been reported to become highly neurotoxic after proteolytic processing by gelatinases in the basal ganglia of mice, leading to neurodegeneration (Zhang et al., 2003). However, we also speculate that non-catalytic hemopexin domain-mediated processes could be involved, so we could also look for the selective expression of integrins that can be engaged to stimulate glutamatergic transmission. Once a potential cofactor is identified, we hypothesize that adding it to the slow Sol or EOM-innervating motor pools in addition to MMP-9 in a mutant SOD1 mouse should be sufficient to induce axonal dieback in these muscles.

The role of the enzymatic activity of MMP-9 in motor neuron degeneration

In Chapter 4 we presented a preliminary attempt to distinguish between the enzymatic and non-enzymatic activity of MMP-9. We showed that mice treated with either a centrally-delivered, specific inhibitor of MMP-9 (MMP-9 Inhibitor I) or a systemically-delivered inhibitor of both MMP-9 and MMP-2 (SB-3CT) exhibited a greater degree of TA muscle innervation as compared to controls at P75. These experiments already suggest that the pathologic mechanism
of MMP-9 involves its enzymatic activity, at least in part. This confirms the conclusions of Lorenzl et al. (2006), who used another inhibitor and reported a modest 13-day survival (discussed more below). However, the protection that we observe is not complete, and this could result either from 1) a role for non-enzymatic mechanisms, or 2) from incomplete enzyme inhibition. As discussed in Chapter 4, all experiments to quantify activity levels have proven unsuccessful thus far. As such, we suggest an additional strategy to test the role of activity.

In Chapter 4, we also presented a robust in vivo gain-of-function assay, which can be used to explore the effects of MMP-9 in vulnerable ALS motor neurons (Figure 4.7). Briefly, when we re-expressed MMP-9 by intramuscular injection of AAV6-Mmp9 cDNA into the protected TA of SOD1<sup>G93A</sup>;Mmp9<sup>-/-</sup> mice, the innervation was reduced to 78 ± 2%. Thus, we propose using this system to determine 1) whether the induced degeneration is MMP-9 specific or whether other MMPs would have similar effects, and 2) whether enzymatic activity and/or the hemopexin domain of MMP-9 is required for axonal dieback or induction of ER stress and protein misfolding at early and late time-points in the disease. In order to do this, we will need additional AAV6 vectors that express either other MMPs, such as MMP-2 or MMP-7, or different forms of MMP-9 that can be administered singly and in combination into the TA muscle of SOD1<sup>G93A</sup>;Mmp9<sup>-/-</sup> mice. The following MMP-9 variants would be useful in determining the mode of action of MMP-9: 1) full-length, catalytically active; 2) full-length, but with the catalytic Glu replaced by Ala, so lacking enzymatic activity; 3) truncated, with a normal active site but deleted hemopexin domain; and 4) active MMP-9, with a mutation of glycine residue 100 to leucine in the prodomain of MMP-9 that would weaken the interaction between the prodomain and the catalytic subunit, inducing the autolytic cleavage and removal of the prodomain. The first 3 of these constructs were initially generated in the lab of
G. Opdenakker, Leuven (Van den Steen et al., 2006) and he generously provided plasmids to our collaborators in Patrick Aebsicher’s group in order to generate the viruses.

Unfortunately, there have been some unexpected setbacks with the virus production. Specifically, the subcloning of the Mmp9 sequences into the pAAV backbone was not successful. In order to troubleshoot what went wrong, Bernard Schneider (Aebischer Lab) sequenced the original plasmids and found that the sequences had been fused in frame with a mellitin secretion signal, causing the restriction sites indicated to be useless for subcloning. The next step taken was to attempt a PCR strategy to generate fragments compatible with their AAV backbone. Again, this failed, invariably producing a 200bp sequence instead of the expected 2.2kb amplicon and attempts to troubleshoot this have also not worked thus far. Our next anticipated step is to get a commercial source to generate the fragments from sequences that can be sent to the Aebischer lab for viral production. At that point, the Aebischer lab can generate the vectors (their turn-around time has been between 4-6 months) and then send them back to Columbia for in vivo testing in our colony.

Though this approach is likely to be more definitive that others, another interesting direction is to further investigate a role of activated MMP-9 and a potential link with NO by determining if and when MMP-9 is S-nitrosylated. There is growing evidence that overproduction of NO can be neurotoxic via aberrant S-nitrosylation of a variety of proteins, which contributes to the accumulation of misfolded proteins, mitochondrial dysfunction, synaptic damage, and neuronal cell death (Nakamura et al., 2013). As discussed previously, MMP-9 is activated by S-nitrosylation when NO reacts with the cysteine residue to expose the catalytic Zn$^{2+}$ in the active site to substrate (Gu et al., 2002). This activation may occur concurrently with other S-nitrosylation events that have also been shown to be harmful to cells. An example
of such an event is that cellular defense proteins such as protein disulfide isomerase (PDI) can be inhibited by S-nitrosylation. Specifically, the formation of SNO-PDI inactivates the chaperone, halting its ability to correct protein misfolding (Jeon et al., 2013; Nakamura et al., 2013; Uehara et al., 2006), which aggravates ER stress. This is likely damaging, as pharmacological inhibition of PDI enzymatic activity has been shown to increase the presence of mSOD1 inclusions in mutant SOD1 mice (Chen et al., 2013a) and substantial levels of SNO-PDI are present in human postmortem brains from patients with sporadic ALS (Walker and Atkin, 2011).

Thus, an increase in NO can set off parallel pathways that are potentially harmful: activating MMP-9 and inactivating proteins involved in the maintenance of cellular homeostasis. Importantly, there have been a number of recent advancements in methodologies to quantify S-nitrosylation events in normal or in diseased tissues (Chen et al., 2013b). Therefore, it would be very interesting to characterize the timing of this post-translational modification of MMP-9 in mutant SOD1mice, compared to WT controls.

These experiments will be very important for future analysis on the mechanism of MMP-9 in vivo which remains perhaps the most pressing unanswered question from this work. There are in fact numerous potential mechanisms that we can deduce from existing literature. For example, MMP-9 has long been known to degrade laminin (Giannelli et al., 1997). This interaction has been shown to directly lead to cell death in different cell types. For instance, it was recently shown that injecting an MMP-9 inhibitor into the neonatal hippocampus increased laminin levels and promoted neuronal survival (Murase and McKay, 2012). MMP-9 also has been shown to trigger the death of retinal ganglion cells, likely by degrading laminin in the inner membrane (Chintala et al., 2002; Guo et al., 2005). Moreover, synaptic laminin is required to maintain adult neuromuscular junctions (Samuel et al., 2012). Intriguingly, laminin-alpha4
remains at synaptic basal laminae of extraocular muscles from ALS patients and mSOD1 mice, but disappears from their limb muscle NMJs (Kjellgren et al., 2004; Liu et al., 2011). Therefore, MMP-9 could be acting by directly degrading laminin at the neuromuscular junctions of fast fibers.

Another substrate of MMP-9 that potentially could link it to ALS is profilin-1. Profilin-1 is a small protein that is essential for the polymerization of monomeric G-actin to filamentous-actin (Witke, 2004), and thus axonal integrity. Although profilin-1 is intracellularly localized, it has been shown to be an MMP substrate (Cauwe et al., 2009; Cauwe and Opdenakker, 2010). Mutations in the profilin-1 gene have been shown to cause familial ALS and primary MNs that express mutant profilin have smaller growth cones (Wu et al., 2012). Therefore, MMP-9 may contribute to ALS pathogenesis by degrading profilin-1 and destroying axonal integrity.

Moreover, MMP-9 has also been reported to cleave amyloid precursor protein (APP) (Backstrom et al., 1996; Yin et al., 2006; Yong et al., 2001). APP is a single pass type-I transmembrane protein that undergoes a series of proteolytic processing steps that create multiple bioactive species. Minute differences in species length can translate into dramatic changes in systemic effect (De Strooper and Annaert, 2010; Lange and Overall, 2013). For example, N-terminal fragments of APP can activate the death receptor DR6, which has been shown to be involved in axonal retraction at the NMJ (Nikolaev et al., 2009). Further, DR6 expression is upregulated in motor neurons of ALS post-mortem samples and SOD1G93A mice and blocking via antibody promotes motor neuron survival in vitro and in vivo (Huang et al., 2013). Finally, inactivation of the APP gene in SOD1G93A mice delays muscle denervation (Bryson et al., 2012). Follow-up studies with an N-terminal specific antibody in SOD1G93A mice with and without MMP-9 will be very interesting.
A final substrate of MMP-9 that could be involved in ALS pathology is NG2. NG2 is a proteoglycan that is strongly expressed by oligodendroglial precursors in the ALS spinal cord (Kang et al., 2010). The degradation of NG2 by MMP-9 reduces proliferation of these progenitors and blocking MMP-9 post spinal cord injury allowed for NG2 precursors to mature into oligodendrocytes and enhanced recovery (Liu and Shubayev, 2011). However, it has also been reported that MMP-9 facilitates remyelination by processing NG2 (Larsen et al., 2003), which was demonstrated to have an inhibitory effect on axonal elongation (Dou and Levine, 1994). Further, a very preliminary study we did in collaboration with the lab of Dwight Bergles (JHU) found that Mmp9−/− mice have greater NG2 immunoreactivity in their spinal cords than WT age-mates. However, Mmp9−/− mice also have lower NG2 cell proliferation than WT age-mates. Finally, we found that NG2 cell proliferation increases with age in SOD1G93A animals, and this happens later in SOD1G93A;Mmp9−/− mice. Therefore, the processing of NG2 by MMP-9 may have an effect on ALS, but more work is needed to understand the relationship.

Thus, there are many potential downstream targets of MMP-9 that could contribute to ALS pathology. Once the role of activity of MMP-9 and the timing of these effects are elucidated, a more informed investigation of potentially ALS-relevant substrates can begin. Further, understanding the role of activity could have important implications for the design of therapeutic agents.

Clinical implications of this work

The most exciting aspect of this work is that it demonstrates that MMP-9 is a promising therapeutic target for the treatment of this devastating disease. We have several reasons to make this claim. First, genetically ablating MMP-9 has a large protective effect in mutant SOD1 mice.
The MMP-9 null mice lived 25% longer and had an approximately 80-day delay in the
denervation of the susceptible TA muscle. Importantly, even partial reduction in gene
expression is enough to confer significant benefit and this is also the case when the reduction
was driven postnatally using RNAi. Moreover, so far MMP-9 is the earliest known intermediate
in the disease pathway triggered by mutant SOD1. Finally, we show that commercially available
inhibitors of MMP-9 have protective effects in these mice at an intermediate disease stage. This
is particularly encouraging because the inhibitor treatment began after symptom onset.

However, all of this work was done in a mouse model of ALS with a mutation found in
only about 2% of all ALS cases. An important future direction will be to determine whether
MMP-9 is relevant to non-SOD1 forms of ALS, especially the sporadic cases of ALS which
make up ~90% of all patients. Other groups are working hard to develop additional rodent
models of ALS using other identified mutations, including TDP-43 (Tsao et al., 2012), FUS
(Huang et al., 2011), and C9orf72, and it will be exciting to target MMP-9 in these and look for
protective effects similar to those we observed in the mutant SOD1 mice. There are a few
reasons we suspect that MMP-9 will be relevant to these non-SOD1 FALS cases as well as to
SALS. First, we initially identified MMP-9 in WT motor pools that are selectively resistant to
ALS in both the mSOD1 mouse model (Figure 3.1) and sporadic ALS patients. Further, a very
recent paper which studied a Chinese population showed that the C(21562)T polymorphism in
the MMP-9 gene, which leads to higher Mmp9 promoter activity, is significantly associated with
an increased risk of sporadic ALS (He et al., 2013). This link with human sporadic patients is
very encouraging, especially because our staining of human samples did not show strong MMP-9
expression in lumbar motor neurons (Figure 2.12). We therefore consider the positive results in
mSOD1 mice as highly promising for future studies in ALS patients.
There are a few different strategies that can be taken to assess the therapeutic effects of altering MMP-9 activity or levels in ALS patients, though determining which is optimal awaits future mechanistic insights. First, in order to reduce MMP-9 activity, pharmacological inhibition is a classic strategy. Our data with specific gelatinase inhibitors suggest this is a promising approach. Further, a previous study by Lorenzl et al. in which Ro 26–2853, a large spectrum MMPs inhibitor from Roche that inhibits MMP-1, -2, -3, -7 and -9, was orally administered to mutant SOD1 mice before or after disease onset (starting at P30 or P90), showed that it could extend the survival of transgenic mice, but only when administered early (Lorenzl et al., 2006). However, minocycline, an antibiotic that also inhibits MMP-9, among many other actions including direct effects on apoptosis and inflammation (Kim and Suh, 2009), had very disappointing results in a large Phase III randomized clinical trial (Gordon et al., 2007). In fact, the results suggested that minocycline harms ALS patients; of the 412 patients with ALS studied, those treated with minocycline declined more rapidly than those on placebo. Further, gastrointestinal side effects were reported. The authors speculate that the potential neuroprotective effects of the drug may depend on when in the course of the illness it is given and acknowledged that the majority of patients included in this study were diagnosed and treated a year or more after their symptoms began. It is also possible that MMP-9 activity was not effectively reduced at the dosage given or that a beneficial effect was masked by harmful non-MMP-9 related effects. More specific inhibitors of MMP-9 including newer pharmacological inhibitors or blocking antibodies should be evaluated.

Another interesting, activity-related clinical direction is using MMP-9 activity to control drug release. All drug delivery systems have to meet several requirements to be successful including: high stability, high accumulation at disease target site, efficient drug release at the
right target site that matches drug pharmacodynamics, and tolerability. The encapsulation method for a given compound greatly influences all of these aspects (Andresen et al., 2010). Enzyme sensitive encapsulation systems where over-expressed disease-associated enzymes are utilized to trigger drug release have been an interesting and promising area of research. Mallik, Srivastava and co-workers at the University of North Dakota have pioneered the use of liposomes containing MMP-9-degradable lipopeptides to unmask therapeutic compounds for controlled drug release (Banerjee et al., 2009; Dutta et al., 2011; Elegbede et al., 2008; Nahire et al., 2012; Sarkar et al., 2008; Sarkar et al., 2005). These papers demonstrate the promise for the specific delivery of cytotoxic drugs to cancer cells, which produce high levels of MMP-9. Given the selective expression of MMP-9 that we report in Chapter 2, we propose using a comparable system to selectively target a drug to vulnerable, fast motor neurons, but not others (Figure 6.1). In the illustrated example, tauroursodeoxycholic acid (TUDCA), which has been shown to be neuroprotective in animal and cell culture models of ALS (Thams et al., in preparation), is concentrated into the core of a nanoparticle which can be activated by proteolysis either outside of fast motor neurons or taken up into these cells by endocytosis and released by MMP-9 intracellular proteolysis. This drug delivery system could reduce off-target side effects.

Further, if future studies point to non-enzymatic roles for MMP-9 in ALS pathogenesis, strategies to reduce MMP-9 transcription or translation may be clinically useful. Examples of these include repressors of gene activation (Labrie and St-Pierre, 2013) and antisense oligonucleotide-based technologies. Antisense oligonucleotides (ASOs) are single-stranded DNA oligonucleotides, usually 8-50 nucleotides in length, which target cellular mRNA transcripts via complementary base pairing (Dias and Stein, 2002). The resultant DNA/RNA duplex gets degraded by RNAse H, and the single stranded ASO gets recycled to mediate
multiple rounds of selective RNA degradation. Therefore, a single administration of ASOs can effectively block protein translation. Further, a previous study showed that an ASO that blocks an SMN2 intronic splicing silencer element still promoted approximately 90% SMN2 exon 7 inclusion 6 months after treatment (Hua et al., 2010). Therefore, an ASO against MMP-9 may be as or more effective than AAV knock-down, but could be delivered to adults.

Thus, understanding the mechanism of MMP-9 and using this knowledge to benefit patients is a top priority for future research.

Conclusions

Taken together, this thesis presents data which shed light upon novel aspects of motor neuron biology, define a potential therapeutic target for ALS and suggest potentially exciting new research directions. My results provide proof of concept for the general usefulness of investigating the selectivity of neurodegeneration to learn more about underlying disease mechanisms and how to inhibit them.
Figure 6.1
**Figure 6.1. Understanding MMP-9’s mechanism of action could aid in the drug delivery design of other candidate therapeutics**

Schematic of a system to deliver drugs only to cells with active MMP-9. When a hydrophilic polymer (such as PEG, blue) is attached to a hydrophobic drug like TUDCA (red), a micelle forms in aqueous conditions in order to minimize free energy of the system. The hydrophobic regions coalesce and the hydrophilic regions are exposed externally to the aqueous environment. Nanoparticles such as micelles are readily taken up by cells, and free MMP9 in the cells would cause degradation of the outer core and release of the free drug. This technique has been used to deliver cytotoxic drugs to cancer cells, which also produce high levels of MMPs.
Chapter 7: Experimental Procedures

Transgenic Mice

For expression studies, ChAT-Cre [B6;129S6-Chattm1(cre)Lowl/J] and ROSA-TdTomato reporter [B6;129S6-Gt(Rosa)26Sortm14(CAG-tdTomato)Hze/J] mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and crossed to generate offspring whose motor neurons selectively express TdTomato. Chodl-LacZ mice were obtained from Lexicon Pharmaceuticals. For functional studies, male mice heterozygous for the mutant human SOD1<sup>G93A</sup> transgene (B6.Cg-Tg(SOD1*G93A)1Gur/J Jackson Laboratory) were crossed with the following mice: females with an MMP9 gene disruption (B6.FVB(Cg)-Mmp9<sup>tm1Tvu</sup>/J, Jackson Laboratory) on a C57BL/6J background, homozygous null Sema3e<sup>nlz/nlz</sup> mice (Pecho-Vrieseling et al., 2009) on a mixed background obtained from Dr. Yutaka Yoshida (Cincinnati Children's Hospital Medical Center), and Phd3<sup>−/−</sup> mice on a CD-1 background obtained from Thong Ma (Burke Medical Research Institute), which had been generated originally in the lab of Guo-Hua Fong (University of Connecticut Health center). For all MMP-9 deletion experiments, SOD1<sup>G93A</sup>;Mmp9<sup>−/−</sup> mice were always compared with their contemporaneously produced SOD1<sup>G93A</sup>;Mmp9<sup>+/−</sup> or SOD1<sup>G93A</sup>;Mmp9<sup>++</sup> littermates. The clinical course for the mutant SOD1 mice was monitored weekly. The disease invariably progressed from hindlimb tremor, and reduced splay to gait abnormalities, followed by complete paralysis. End-stage of disease was defined as the age at which mutant SOD1 mice could no longer right themselves for 30 seconds after being put on their side. All animal work was performed in compliance with the Columbia University IACUC protocols.
Immunohistochemistry

Mice were deeply anesthetized using 3.5% avertin and transcardially perfused with 10 mL of ice cold phosphate-buffered saline (PBS) followed by 50 ml of 4% paraformaldehyde in PBS. Muscles were washed in PBS overnight, and spinal cords and brains were post-fixed in the 4% paraformaldehyde overnight and then all were processed in a sucrose gradient for cryoprotective embedding. For visualization of the NMJ in various whole muscles, α-bungarotoxin conjugated to tetramethylrhodamine (TMR), Alexa fluor 488, or Alexa fluor 647 (Invitrogen, Carlsbad, CA, Carlsbad, CA) 1:500 was added with rabbit anti-VACHT 1:2000 (Sigma-Aldrich, St. Louis, MI) or rabbit anti-VACHT 1:32,000 (Henderson Lab via Covance, Denver, PA). For analysis of motor groups in the brainstem and spinal cord, the following primary antibodies were used: goat anti-ChAT 1:100 (Millipore, Billerica, MA), rabbit anti-MMP-9 1:4000 (Abcam, Cambridge, MA), goat anti MMP-9 1:2000 (Sigma-Aldrich, St. Louis, MI), rabbit anti-GFP 1:1000 (Invitrogen, Carlsbad, CA), mouse anti-NeuN 1:600 (Chemicon, Billerica, MA), rabbit anti-phosphorylated EIF2α (Ser51) 1:150 (Cell Signaling Technology, Inc. Beverly, MA), rabbit anti-calreticulin 1:1000 (Thermo Fisher Scientific Inc, Rockford, IL), and chicken anti-β-galactosidase 1:1000 (Abcam, Cambridge, MA). Secondary antibodies conjugated to Alexa fluor 488, 555, and 594, and 647 were generated in Donkey (Invitrogen, Carlsbad, CA). Images were acquired on a Nikon Eclipse TE-2000-E fluorescent microscope. The protocol was modified slightly for p-EIF2α staining; slides were blocked in 3% BSA, 0.3% triton X and the primary antibody remained on the slides for at least 48 hours. Staining was always performed alongside a negative control so that when imaged the exposure could be adjusted to the control levels to reduce background.
In situ hybridization

Tissue preparation was performed as described above and in-situ hybridization protocol was followed as previously described (Arber et al., 2000) on tissue from wild-type, SOD1\textsuperscript{G93A} mice, and Mmp9\textsuperscript{+/-} mice, all on pure C57BL/6J background. Probes were generated from cDNA vectors purchased from Open Biosystems Inc. (Huntsville, AL). These included the following OpenBiosystem (clone ID numbers are in parentheses): Mmp9 (6309245), Egln3 (6528802), Mmp2 (6813184), Timp2 (6821553), Timp3 (4239015), and Chodl (40126392). Chat and Sv2a were previously generated in the Henderson laboratory by Kevin Kanning. Vectors contained either full-length cDNA sequences or the sequences corresponding to the 5’ UTR regions of genes of interest flanked by SP6, T3, or T7 promoters. For each of these, clones were first linearized at the 5’ end of the cDNA sequences with restriction enzymes and used as a templates for synthesis of antisense RNA probes using either T7, SP6, or T3 polymerases, depending on which of the promoters was located to the 3’ of the cDNA sequence, and digoxigenin (DIG)-labeled UTPs (Roche Applied Science, Indianapolis, IN). Successful synthesis was verified by running the labeled RNA probes on 1.2 % agarose gels to check for appropriate sizes.

Retrograde labeling

Cholera Toxin Subunit B conjugated to Alexa fluor 488 or 555 was injected was injected into the Sol, TA, or GC muscles of WT or Mmp9\textsuperscript{+/-} mice at P5 or P40. Animals were perfused and spinal cords were collected 48-96 hrs later. At that time, the injected muscle and surrounding muscles were checked for fluorescence to assure specificity of injections.
Behavioral assays

Mice were assessed for motor function using the accelerated rotarod task twice weekly from P40 onward. Briefly, we weighed each mouse before the trial and then placed animals on an accelerating rod (4-40 rpm over a 5 minute period) and recorded the time it took for each mouse to fall from the rod. We performed 2 trials at each time point for each animal and recorded the longest time taken to fall and used it in analyses. A decrease in time on the rotarod was observed with increasing motor impairment.

We also used a weekly swim tank test to assess progression of motor deficit from P40 onward. The test was constructed as per the specifications of Raoul et al. with some slight modifications (2005). Test subjects were lowered individually into one end of a 75 cm long x 6 cm wide x 40 cm high Plexiglas container filled partially with 20°C water and induced to swim to a ramp at the other end that protruded into the water. Footage of each trial was recorded using a video camera. Each subject underwent a minimum of five trials, with the shortest time excluded from analysis and the next three shortest times averaged to obtain a value for that individual at that timepoint. Length of swim was determined from when the hindlimbs first contacted the surface of the water to their first contact with the ramp. A progressive slowing of an animal’s swim rate was considered a signal of motor dysfunction.

Electrophysiological measurements

Mice were anaesthetized with inhalational isoflurane. Two stimulating electrodes were placed at a paraspinal site on both sides of the sciatic nerve. A controlled stimulation was applied to the nerve in 10 to 50 µA increments from 50 µA to evoke and record unitary and maximal isometric
twitch and tetanic isometric contractions from each muscle. The M-wave was measured at each escalating amplitude, until the maximal response was elicited and did not increase further.

**Electrical impedance myography**

All EIM measurements were performed as previously reported (Li et al., 2013). The animals were anesthetized with isoflurane delivered by nosecone with a heating pad underneath the limb to maintain a consistent temperature. After the fur overlaying the left GC muscle was clipped, a depilatory agent was applied to the skin to remove all remaining fur and the skin was then cleaned with saline. The impedance measuring system consisted of a multi-frequency lock-in amplifier (Model 7280, Signal Recovery, Oak Ridge, TN) coupled with a very low capacitance active probe (Model 1103 of Tektronix, Beaverton, OR) providing data from 0.5-1000 kHz. The two parameters obtained from this system are resistance \( R \) and reactance \( X \) from which the third parameter, phase \( \theta \) is calculated via the relationship \( \theta = \arctan(X/R) \).

**Experiments with viral vectors**

*Generation of AAV6 vectors from Aebisher lab:* The following sequences were subcloned into the AAV6:shSOD1 vector (Towne et al., 2008) replacing the shRNA sequence targeting mutant SOD1 with TRC31231 under the H1 promoter:

- **AAV6:shMmp9**: the TRC31231 clone expressing a shRNA sequence
- **5’GAGGCATACTTGTACCGCTAT3’** targeting nt 145 – 165 in the coding region of *Mmp9*
- **AAV6:mismatch**: an off-target shRNA control vector containing an shRNA insert that does not target human and mouse genes (SHC002)
Both were purchased from Sigma Aldrich (St. Louis, MI). AAV6 virus was produced and concentrated as previously described (Towne & Aebischer, 2009). Viral titers for AAV6:U6-sil MMP9 (trc31) were $2.1 \times 10^{14}$ vg/ml and AAV6:U6-mis MMP9 were $2.3 \times 10^{13}$ vg/ml.

Other vectors: All self-complementary vectors were designed and made by Guangping Gao (University of Massachusetts Medical School) and mailed overnight on dry ice to avoid freezing and thawing. rAAV6/TR-eGFP was generated by David Dismuke (University of North Carolina) at a titer of $3 \times 10^{12}$ vg/mL.

Vector administration: For intracerebroventricular (i.c.v) injections, P1 pups were anesthetized by hypothermia by placing them on wet tissue overlaying the ice for 2 minutes. Pups were placed ventral side down onto the metal stage that was previously cooled on ice and stabilized with scotch tape. To identify the injection site on the skull a diagonal from lambda to the eye was traced. A point 1 mm away from the midpoint toward lambda was marked. Using 31 Gauge 1/2 cc Insulin syringe (BD Ultra-Fine* II Short Needle Insulin Syringe, VWR) 2 µl of virus was injected at the marked point into the third ventricle. 3mm was the maximum depth the syringe was allowed to penetrate the skull. Virus solution was colored by addition of a small amount of Fast Green dye (Sigma-Aldrich, St. Louis, MI). Success of the i.c.v injections was monitored by transilluminating the head of the injected pup to assess whether the dye spread to both ventricles. If the dye did not spread to both ventricles, the injections were deemed unsuccessful and the pups were sacrificed. For intramuscular (i.m.) injections, P4 pups were anesthetized by hypothermia. The skin overlaying the TA muscles was opened and 1 µl of the AAV6.shMMP9 virus was injected using an insulin syringe. Following that injection, the pup was flipped and the contralateral TA muscle was injected with 1 µl of the AAV6:mismatch virus. Successfully
injected pups were allowed to recover for 5 -10 min on the warming blanket before being returned to the mother.

*Tissue Homogenization*

Mice were perfused with ice cold PBS and lumbar ventral spinal cord fragments, muscles, spleen, and placenta were rapidly dissected and snap-frozen in liquid nitrogen and stored at -80°C. The fragments were then extracted for 1 h on ice using 50 mM Tris-HCl, pH 7.4, containing 1% Triton X-100, 0.2 M NaCl, 10 mM CaCl₂, the proteinase inhibitor cocktail set III (Calbiochem) and 1 mM phenylmethylsulphonyl fluoride. The protein concentration of the extracts was measured using a Coomassie Protein Assay (Thermo Scientific, Waltham MA) and then adjusted to equal 2 mg/ml each. The extracts were then allowed to bind gelatin Sepharose-beads overnight at 4°C. After extensive washing, the bound material was eluted using 2×SDS gel loading buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 0.005% Bromophenol Blue and 20% glycerol).

*Gel Zymography*

Gel Zymography was performed using Novex Zymogram Gels containing 0.1 % gelatin (Invitrogen, Carlsbad, CA) following manufacturer’s protocol. Briefly, recombinant MMP-9 and MMP-2, tissue homogenates, or conditioned media from macrophage cultures were loaded in Novex Tris-Glycine 2x SDS sample buffer (Invitrogen, Carlsbad, CA) onto 10% zymogram (gelatin) gels (Invitrogen, Carlsbad, CA). Proteins were resolved by electrophoresis. Gels were washed in zymogram renaturing buffer to remove SDS (Invitrogen, Carlsbad, CA), followed by incubation in the zymogram developing buffer (Invitrogen, Carlsbad, CA) overnight at 37°C.
Gels were rinsed with water and stained for 1 hour with Coomassie blue (SimplyBlue SafeStain, Invitrogen, Carlsbad, CA), followed by destaining with water. Areas of gelatinolytic activity appeared as clear bands. After destaining the gels were scanned. Digital images were processed using Image J software (National Institutes of Health, Bethesda, MD) for densitometric analysis.

**In Situ Zymography**

For *in situ* zymography, mice were perfused with ice cold PBS and lumbar ventral spinal cord fragments, muscles, spleen, brain, and placenta were rapidly dissected and placed in a mold filled with OCT compound (Tissue-Tek) and frozen on crushed dry ice. Cryostat sections were cut at variable thicknesses between 8 µm and 40 µm and allowed to dry on coated slides. Slides were then treated with one of the following fixatives (or, in the “no fix” condition, just washed with PBS): acetone for 10 minutes at -20° C, 100% methanol for 10 minutes at -20° C, 70% ethanol for 10 minutes at 4° C, or 4% PFA for 10 minutes at 4 degrees. Slides were washed 2 X 5 minutes with PBS + 0.1% Triton X and were then either blocked first at room temperature for 1 hour with 1% BSA or were immediately incubated at 37° C with 10 µg/mL or 100 µg/mL DQ gelatin fluorescin conjugate (EnzChek Gelatinase Assay kit; Invitrogen) for 2 hours.

**Pharmacological Inhibition**

*MMP-9 Inhibitor 1:* WT or SOD1<sup>G93A</sup> mice (18-22 g) were anesthetized with isoflurane at P50 and placed in a stereotaxic instrument and a guide cannula (Plastics One, Inc., C311GS-4/SP with tubing length below pedestal of 3 mm) with a dummy inserted in it to prevent clogging when not in use (Plastics One, Inc., C311DCS-4/SP to fit 3mm without projection) was placed 1 mm lateral and 1 mm caudal to bregma, at a depth of 2.5 mm. The guide cannula was secured
to the skull using mounting screws (Plastic One) and dental cement (Dental Products of India, Mumbai). The animals were allowed to recover for 5 days and then given daily injections into their cannulae of MMP-9 Inhibitor I (2 µg per day dissolved in 10% DMSO, Calbiochem) or a vehicle. The volume of all i.c.v. injections was 10 µl, using a 25 µl syringe (Hamilton Co., Reno, NV, USA) connected to internal cannula (26 gauge, Plastics One, Inc, C311IS-4/SP to fit 3 mm with a 0.1 mm projection) by polyethylene tubing. Animals were perfused after 20 days of injections, at P75.

**SB-3CT:** Two groups of animals were treated with SB-3CT (EMD Biosciences, San Diego, CA) (25 mg/kg, i.p.) or vehicle (10% DMSO) from either P35-P50 or from P55 to P75. Mice were weighed daily before drug administration for dosing and also to measure potential weight loss and/or other negative health outcomes. The younger group of animals were given drug holidays after every 5 days of treatment, and therefore had fewer total injections than the older group of animals (13 vs. 21 injections). Animals were sacrificed at either P50 or P75 and TAs were harvested for innervation analysis.

**Human tissue immunostaining**

Postmortem human spinal cord tissues were obtained from the New York Brain Bank (Columbia University, New York) and were paraffin embedded and sectioned at 8 µm. Routine H&E and antibody staining using rabbit anti-human MMP-9 (Prestige collection, Sigma) were performed by the immunohistochemistry laboratory in Columbia University’s Department of Pathology.

**Anatomical and Statistical analyses**

For the quantification of VACHT and BTX staining of muscle cryosections, consecutive longitudinal 30-40 µm cryosections of muscles were stained with VACHT and BTX. For large
muscles (TA, Sol, IC, BC, EAS, EUS) this analysis was performed on every second or third section. For the extraocular muscles, every section was used. Overlap was most often counted manually, but occasionally also by using the NIS-Elements 3.0 software (Nikon Instruments Inc, Melville, NY) to process the images. For this type of processing, a region of interest was drawn manually around the synaptic band. For each image, pixel intensity in the region of muscle outside the synaptic band in each channel was sampled and designated as background. Binary thresholding was performed to identify discrete areas of pixel intensity above background levels. These areas were counted in both VACHT and BTX channels. Areas of overlap between the two channels were identified and counted. %NMJ innervation was determined by dividing the total number of areas of overlap between VACHT and BTX signals (total number innervated endplates) by the number of areas containing BTX signal (total number of endplates).

For the quantification of motor neuron numbers, motor neurons were counted in consecutive transverse 20 μm cryosections of the L5 and L6 levels of the spinal cord from age- and sex-matched wild-type littermate controls stained for ChAT. A 2-mm extent of the L5-S1 spinal cord (67 sections) was sampled. In each animal, the cryosection corresponding to the L6/S1 border region was identified by visual inspection of the disappearance of the DL nucleus and the prominent V nucleus. The L6 level on each side was defined as the 1-mm region rostral to the L6/S1 border section. Motor neurons in this region were counted in every consecutive 20 μm section. DL motor neurons were identified by their characteristic location at the lateral border of the ventral horn and the characteristic positions. Motor neurons located dorsally to this population were considered to be RDL motor neurons. For each animal numbers of motor neurons in L5, RDL, and DL in left and right ventral horns were then summed.
For the quantification of MMP-9 fluorescence intensity, staining for MMP-9 and ChAT was performed on 20-μm cryosections of the spinal cord and 30-μm free-floating cryosections of the brainstem. Counts of MMP-9+ and ChAT+ motor neurons were performed in every fifth section in left and right ventral horns (10 sections total per animal). Oculomotor, trochlear, and abducens nuclei were located in coronal sections of brainstems based on the published rostrocaudal positions in adult brainstem as previously described (Ferrucci et al., 2010). Counts were performed in six sample sections from the entire rostrocaudal extent of the oculomotor nucleus spaced at 150 μm and in two and three sample sections spaced at 60 μm for trochlear and abducens nuclei, respectively.

The two levels of MMP-9 staining intensity designated as MMP-9hi and MMP-9lo were clearly distinguished. Only cells with ChAT+ cytoplasm surrounding a clearly visible nucleus were counted. To quantitatively confirm manual counts of MMP-9hi and MMP-9lo motor neurons, ImageJ software (National Institutes of Health, Bethesda, MD) was used to process images acquired for L5 motor neurons in one wild-type animal (273 motor neurons counted). A 5-μm² area within the cytoplasm of each ChAT+ motor neuron was manually picked. The summed fluorescence intensity in the MMP-9 channel for each area was then calculated. To eliminate variation between images, a background, non-ChAT+ point was also selected and the summed intensity from this region was then subtracted from all other intensity values from that image. The data were then sorted by intensity and plotted using Excel. A best-fit trendline, determined by maximizing the R² value, was then added and the second derivative was taken to find the inflection points of the function. The ordinate values of the two inflection points were considered.
to be the bin threshold. Specifically, all cells with an intensity lower than the first inflection point were considered MMP-9\textsuperscript{neg}, all cells between the two inflection points were considered MMP-9\textsuperscript{lo} and all cells above the second inflection point were considered MMP-9\textsuperscript{hi}. The number of cells in each category was then compared to manual counts.

For motor neuron cell size measurements, the largest cross-sectional areas were determined using NIS-Elements 3.0 software (Nikon Instruments Inc, Melville, NY) for cells with a ChAT\textsuperscript{+} cytoplasm with a visible nucleus. All ChAT\textsuperscript{+} MNs with a visible nucleus were imaged at 10x and outlined. Distribution histograms were constructed for each level of MMP-9 staining intensity, by grouping cell body cross-sectional areas in 100-\(\mu\text{m}^2\) bins.

For CRT intensity measurements, CRT immunofluorescence was measured in the spinal cord sections from P50 SOD1\textsuperscript{G93A};Mmp9\textsuperscript{-/-} mice (n=3 animals) that had been injected into one TA at P4 with AAV6.MMP9. For all alpha-MNs (identified by NeuN positivity), the pixel intensity was measured using Image J software (National Institutes of Health, Bethesda, MD). After intensity measurements were taken from the channel with CRT fluorescence, the channel was switched to the channel with MMP-9 in order to characterize the MN into one of the following categories: MMP9-positive MNs, MMP9-negative MNs from the injected ventral horn, MMP-9 negative MNs from the uninjected side (65-100 MNs each). The resultant categories had unequal variance, so a non-parametric statistical analyses, so were compared using either a Kruskal-Wallis One Way Analysis of Variance on Rank or a Mann-Whitney Rank Sum test, depending on if the “injected side” categories were combined or not. WT mice that had been
given TA injections of AAV6.GFP were used as a control for potential changes in CRT fluorescence resulting from viral transduction.

Tests of significance between two genotypes were conducted using an unpaired Student’s t-test. A Student’s t-test was also used to for the p-EIF2α and MMP-9 co-localization analysis. Specifically, the probability of cells being co-positive by chance was calculated (#MMP9 - positive MNs/ total MNs * #p-EIF2α-positive MNs/ total # MNS), and that number was compared to the observed population of co-positive cells (co-positive cells/ total # MNs) for each animal. Tests between three or more groups were conducted using a one-way ANOVA with a Student Newman-Keuls post hoc analysis. For analyses of skin facing versus bone-facing side denervation in TA muscles, data were analyzed by two way ANOVA, with the 2 factors designated as time and fiber type. Post-hoc pairwise comparisons were made by the Holm-Sidak method. Survival analyses were generated by Kaplan-Meier estimates with a log rank analysis. All calculations were performed using SigmaPlot software. All α-thresholds were set at 0.05 unless otherwise specified.
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