A Study of the Mechanism of Motor Neuron Death in Amyotrophic Lateral Sclerosis

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ABSTRACT

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Amyotrophic Lateral Sclerosis (ALS) is a fatal adult-onset paralytic disorder for which there is currently no cure. Underlying the disease mechanism of ALS is the spontaneous pathologic degeneration of motor neurons (MNs). Understanding the molecular mechanisms underlying spontaneous and selective MN demise is critical to the development of rational therapeutic strategies. In the current work, utilizing established in vitro models of ALS, I demonstrate that necroptosis, a form of caspase-independent programmed cell death (PCD), drives MN death. Pharmacologic inhibition and/or genetic silencing of receptor interacting protein kinase-1 (RIPK1), receptor interacting protein kinase-3 (RIPK3), and mixed lineage kinase domain-like-protein (MLKL) rescued MN death in vitro. While this core machinery was conserved, the requirement of nuclear factor kappa-B (NF-κB) and Bcl-2-associated X protein (Bax) deviated from known models of necroptosis. This divergence led me to consider that there may be a MN-specific program of necroptosis. Thus, I then used unbiased approaches, by meta-analyzing a gene expression signature captured from MNs undergoing cell death in vitro, to explore MN cell death drivers that may be engaged upstream or downstream to RIPK1/RIPK3/MLKL. I also explored the relevance of necroptosis to MN disease in vivo, in part by deleting RIPK3 from a genetic mouse model of familial ALS. Overall this approach did not rescue motor neuron loss, and there was no improvement in motor function, disease onset, or survival in these animals. I conclude that while necroptosis machinery drives motor neuron death in in vitro models of ALS, more work needs to be done to (1) assess the motor neuron-specific cell death program, and (2) evaluate the relationship, if any, of necroptosis to motor neuron disease in vivo.
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Chapter 1: Introduction

As a biologist, I have always been struck by the elegance of complex signaling systems between cells of a multicellular organism, which integrate to create a working machine, e.g., the human body. One process that is imperative for the normal development of a multicellular organism is developmental cell death. This programmed cell death is ordered, deliberate, selective, and accounts for errors in redundancy. For example, the mammalian nervous system creates many more neurons than will survive and mature, and selects to survive and mature those neurons that pattern the correct connections. Other developmental programs more directly result from evolution, as in the classic example of selective death of cells in the connective tissue of webbed hands that generate digits during human development.

While this elegant, programmed and systematic process of selective cell death is so critical to normal development, the questions arise: What happens in diseases in which cell death itself is a consequence, such as in neurodegenerative disease? Is this cell death a controlled, deliberate process, as in normal development, or is it a passive result of a pathological insult that has acutely changed the surrounding environment to become unsupportive of cellular integrity?

One cannot begin to discuss cell death without first introducing the historically-defined dichotomy between apoptosis, e.g., molecularly-controlled programmed cell death, that accounts for developmental programs, and necrosis, which occurs by a passive process in which cells are overcome by a stressful injurious event in their environment, such as sharp change in pH, which even if it were possible, would leave no opportunity to integrate a response through regulated signaling.
For decades, it was assumed that pathologic cell death, occurring as a result of disease, was a passive process resulting from necrosis and that only developmental cell death was molecularly-controlled via apoptosis. In diseases in which cell death is a major consequence, such as in neurodegenerative diseases, this notion leaves little room for conventional treatment options that often target a key regulator involved in aberrant signaling.

In recent years, significant advances in our understanding of cell death processes and mechanisms of neurodegenerative disease have been made. Starting about 20 years ago, there was growing emphasis on the idea that the mechanisms driving the death of neurons in neurodegeneration may not be passive as it was once thought, but rather the result of, at least in part, programmed cell death. Several previous works by my mentor, Dr. Serge Przedborski, were of critical influence in such discussions. Attempts to target classical apoptosis mechanisms showed marked but quite modest effects on disease models of neurodegeneration. While promising, it was clear that targeting apoptosis did not mitigate the disease to an extent that would be therapeutically relevant. Excitingly, in the past decade, advances in our understanding of cell death mechanisms have challenged the dichotomy between apoptosis and necrosis. Indeed, an explosion of intermediate, alternative mechanisms have recently been uncovered, which rely on signaling molecules that may, in part, conserve some elements of apoptosis, but do not follow classical execution mechanisms; these alternative mechanisms may have morphologic criteria more consistent with necrosis, which, before modern advances in molecular biology, would have been characterized as such.

As I began my studies in the Pathobiology and Molecular Medicine program, I was struck by the sheer selective nature of cells affected by neurodegeneration. Indeed, in each condition,
only a discrete subset of neurons is affected, which defines how we categorize unique diseases and their pathological hallmarks. In joining the Przedborski lab, my goal was to embark on a series of studies, guided by our advances in the current understanding of cell death and neurodegenerative disease, to investigate the nature of neuronal cell death occurring in this curiously cell-type selective manner in neurodegeneration. I chose to focus my studies on Amyotrophic Lateral Sclerosis (ALS), a disease model for which many in vitro and in vivo experimental systems have been created, and numerous causative inheritable genetic mutations have been identified in recent years. Yet for ALS, the mechanism of selective neurodegeneration in this fatal disease still remains obscure.

1.1. General Introduction to ALS

As reviewed by Rowland and collaborators (2010), ALS is an adult-onset disorder that manifests clinically within the fifth to sixth decade of life and is characterized by progressive paralysis. There is currently no cure for ALS, and the disease is typically fatal within 3-5 years after symptom onset, with patients succumbing to respiratory failure. In fact, merely 10% of patients survive more than 10 years after diagnosis. Until recently, the only known pharmacologic agent approved to treat ALS patients is the drug riluzole, approved in the US in 1995, which only extends survival by 2-3 months. However, the mechanism of action for this drug in extending ALS patient survival remains unclear. Recently, edaravone (Ito et al., 2008, FDA press release; May 5, 2017) was approved to treat ALS, which is again not curative, but clinical data demonstrated that the drug slows the progression of motor symptom decline.
ALS is a neurodegenerative disease of the central nervous system (CNS) in which the pathophysiology of the onset and progression is driven by retraction of motor neuron axons from nerve terminals at the neuromuscular junction and the subsequent loss of upper and lower motor neuron cell bodies in both the brain motor cortex, brainstem and spinal cord. As a result, the critical connections between the CNS and skeletal muscles are progressively lost, resulting in paralysis and eventual respiratory failure and death (Munsat et al., 1988; Schwartz and Swash, 1995).

The overall incidence of ALS in the US is about 4 new cases per 100,000 people (Mehta et al., 2016). Because of the high mortality rate, the relative prevalence is close to the number of new cases each year; an estimated 15,000 people currently live with ALS in the US, which has a population of just over 300 million (Mehta et al., 2016).

### 1.1.1. Clinical Features and Diagnosis of ALS

The diagnosis of sporadic ALS, in which the cause is unknown, occurs as a diagnosis of exclusion when other possible causes of the observed symptoms are ruled out and monitoring detects progression of the disease (Swinnen and Robberecht, 2014). Most patients present with an asymmetric weakness in a limb in the fifth to sixth decade of life. One common initial presentation is weakness in fine motor skills in a finger, affecting the patient’s ability to perform an ordinary task, thus termed limb or spinal onset ALS (Figure 1.1, reproduced from Swinnen and Robberecht, 2014).
The majority of cases are spinal onset, where symptoms begin in the arms and the legs. However, bulbar onset, in which slurred speech and difficulty swallowing are some of the initial presenting symptoms, makes up fewer cases (20-25%) but is more progressive and more severe (Figure 1.1) (Al-Chalabi et al., 2016; Swinnen and Robberecht, 2014).

Patient performance on neurological exams, EMG (electromyography) and nerve conduction studies are carefully monitored over time. A diagnosis of ALS can be determined by criteria that support both upper and lower motor neuron impairment and involvement of at least three regions of the body; the bulbar region and at least two spinal regions or in three spinal regions (de Carvalho et al., 2008). A summary of upper and lower motor neuron signs is detailed in Figure 1.2., reproduced from Swinnen and Robberecht (2014). Signs of upper motor neuron involvement include Babinski signs, hyperreflexia, spasticity, pseudobulbar affect and Hoffman signs. Signs of lower motor neuron involvement consist of muscle atrophy, weakness and fasciculations.

ALS is progressive and most patients succumb to the disease within 3-5 years after initial presentation. While early symptoms manifest as muscle weakness and cramping,
fasciculations, spasticity, slurred speech and difficulty chewing or swallowing, these quickly progress into more pronounced weakness and atrophy, dysarthria, dysphagia, and hyperreflexia.

It should be noted that the clinical features of sporadic and inherited ALS are indistinguishable. However, an earlier age of onset (by about 10 years) and/or family member with ALS typically warrants genetic testing for heritable ALS-linked genetic mutations, further elaborated on in section 1.1.2.

It should also be noted that a subset of ALS patients also experience frontotemporal dementia (ALS-FTD). The co-occurrence of the two diseases in subsets of patients, which share common neuropathological features, is an area of ongoing exploration with the identification of shared genetic etiologies within families. The discussion of ALS and ALS-FTD co-occurrence is beyond the scope of the thesis but contributes greatly to our understanding of the mechanisms by which distinct neurologic diseases, in this case along an ALS-FTD axis, can result from shared genetic factors (Guerreiro et al., 2015).

### 1.1.2. Genetic Mutations in Inherited and Sporadic ALS

At least 90% of ALS cases are sporadic with no known familial inheritance pattern (Taylor et al., 2016). The other 10% of cases can be attributed to familial inheritance of a number of genetic mutations (Figure 1.3). Since the first mutation in families was identified in 1993 by sequencing of the *SOD1* gene (Rosen et al., 1993), upwards of 20 genetic loci linked to ALS have been identified [Table 1.1, reproduced from (Kirby et al., 2016)]. Most notably, about a third of familial cases can be attributed to mutations in *C9orf72*, a hexanucleotide repeat expansion, and about one-fifth of familial cases result from mutations in *SOD1* (Figure 1.3) (Kirby et al., 2016; Taylor et al.,
Interestingly, the majority of these mutations are inherited in an autosomal-dominant manner, in that only one inherited allele containing the mutation is sufficient for the development of ALS.

Although most sporadic cases with no inheritance pattern remain of unknown etiology, recently it was found that in at least 12% of sporadic cases, *de novo* mutations in familial-associated genes can be linked to the disease, with mutations in *C9orf72* accounting for the vast majority of sporadic ALS cases (Kirby et al., 2016; Taylor et al., 2016). Figure 1.3 details the relative contributions of the more common genetic loci identified in both familial and sporadic ALS to date, and Table 1.1 [reproduced from Kirby et al. (2016)] shows the list of known mutations.
Table 1.1. Overview of key information available for ALS and FTDALS loci

<table>
<thead>
<tr>
<th>Loci</th>
<th>Method</th>
<th>Chromosomal location</th>
<th>Gene</th>
<th>FALS/ALS frequency (%)</th>
<th>Year</th>
<th>Onset</th>
<th>Inheritance</th>
<th>Implicated pathogenic mechanisms</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS1</td>
<td>Linkage</td>
<td>21q22.11</td>
<td>SOD1</td>
<td>Range</td>
<td>1993</td>
<td>Adult (AR)</td>
<td>AD (AR)</td>
<td>Oxidative stress, UPS, autophagy</td>
<td>Rosen et al.6</td>
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<td>AD (AR)</td>
<td>Endoskeletal trafficking</td>
<td>Andersen et al.36</td>
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<td>Not available</td>
<td>2002</td>
<td>Adult AD</td>
<td>AD (AR)</td>
<td>RNA processing, stress granule function</td>
<td>Hidano et al.75</td>
</tr>
<tr>
<td>ALS4</td>
<td>Linkage</td>
<td>9q34.13</td>
<td>SETX</td>
<td>Not available</td>
<td>2020</td>
<td>Juvenile AD</td>
<td>AD (AR)</td>
<td>Intracellular cargo transport, axonal growth</td>
<td>Chen et al.37</td>
</tr>
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<td>ALS5</td>
<td>Linkage, candidate gene, WES</td>
<td>15q21.11</td>
<td>SPG11</td>
<td>40%</td>
<td>2004</td>
<td>Adult AD</td>
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<td>RNA processing, stress granule function</td>
<td>Orlacchio et al.37</td>
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<td>Linkage, candidate gene</td>
<td>16p11.2</td>
<td>FUS</td>
<td>4%</td>
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<td>Adult (AR)</td>
<td>UPR, ER stress, intracellular membrane trafficking</td>
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Table reproduced from Kirby et al. (2016) Deg. Neurol. and Neuromus. Dis. 6: 49-64.
1.1.3. Neuropathology Observed in ALS

1.1.3.1. Cell Types Showing Pathology in ALS

ALS is characterized primarily by the degeneration of both upper motor neurons of the motor cortex, brainstem nuclei, and lower motor neurons of the spinal cord anterior horn, with loss of axons in the lateral columns of the spinal cord. Additionally, glial cells show observable signs of reactive gliosis (Schiffer et al., 1996), not as a primary cause but rather as an inflammatory response to the degeneration of the neighboring motor neurons, concomitant insults to the neurovascular unit and breakdown of the blood brain barrier (Burda and Sofroniew, 2014).

1.1.3.2. Neuropathological Features of ALS

A number of neuropathological features have been observed in the central nervous system tissue from ALS patients, the most common being cytoplasmic inclusions in motor neurons. These inclusions are subcategorized by their morphology, reaction with various histologic stains, and by immuno-reactivity for associated proteins. These include Bunina bodies and skein-like cytoplasmic inclusions (Saberi et al., 2015).

1.1.3.2.a. Bunina bodies

Bunina bodies, which are ubiquitin-negative inclusions, are observed in at least 85% of all ALS cases and are most commonly found in motor neurons of

![Figure 1.4. Bunina bodies positive for cystatin C indicated by arrows are present in sporadic ALS patient sALS (left) but not in control (CTL) (right) spinal cord tissue. Image reproduced from Saberi et al. (2015).](image-url)
the spinal cord and in the brainstem nuclei. They appear as 3-5 µm eosinophilic ovals or in chain-like structures with hematoxylin and eosin staining and have shown immunoreactivity for cystatin c, transferrin, and peripherin proteins (Saberi et al., 2015) (Figure 1.4).

1.1.3.2.b. Skein-like inclusions

The other inclusions, called Skein-like, are the most common inclusions observed in ALS occurring in at least 90% of patients. Skein-like inclusions are transactivation response DNA protein-43 (TDP-43)-positive (Saberi et al., 2015), but can be positive for fused in sarcoma protein (FUS) or SOD1. Skein-like inclusions are ubiquitin-positive cytoplasmic inclusions 15-20 nm in diameter that have a filamentous
appearance (Figure 1.5) (Saberi et al., 2015). TDP-43-positive and FUS-positive inclusions also occur in frontotemporal dementia, the discovery of which greatly contributed to the notion that a subset of patients can experience symptoms which occur along an ALS-FTD axis (Neumann et al., 2006).

1.1.4. Links Between ALS Genetics, Neuropathology, and Disease Mechanism

The question as to whether differences in ALS genetics and pathology lead to distinct subclasses of the disease that involve distinct mechanisms is yet to be determined. Importantly, we have learned that the historic categorization between sporadically-inherited vs. familially-inherited ALS does not seem to delineate distinct mechanisms as there are no distinctions in patient presentation. Mutations in common genetic loci are shared between familial and sporadic ALS, due to de novo mutations in sporadic patients.

Rather, subclasses of ALS can be differentiated by unique neuropathology, such as TDP-43-positive neuropathology and FUS-positive neuropathology, which occur in both FTD and ALS. TDP-43-positive neuropathology is not seen in SOD1-related ALS that has not been linked to FTD (Mackenzie et al., 2007; Saberi et al., 2015). A majority of ALS cases show TDP-43 pathology (Mackenzie et al., 2007; Saberi et al., 2015). Additionally, some ALS-associated genetic mutations can be grouped categorically into subsets by similar proposed mechanisms, select examples of which are briefly summarized below.
1.1.4.1. RNA Transport and Metabolism

One mechanism that has been proposed as the functional consequence of genetic mutations linked with ALS is the disruption of normal RNA transport and metabolism. ALS-linked mutations that may lead to this common mechanism include (1) T ARDBP (encoding TDP-43), (2) FUS, in which gene products are known RNA binding proteins, and (3) C9orf72 (DeJesus-Hernandez et al., 2011), in which the RNA gene product resulting from the hexanucleotide repeat expansion has been shown to form RNA foci that could sequester RNA binding proteins and disrupt their normal functions. Indeed, motor neurons seem to be particularly susceptible to defects in RNA metabolism. In spinal muscular atrophy, for example, there is a gene dosage effect for survival motor neuron (SMN), an RNA binding protein with known roles in splicing, in relation to the severity of the disease (Burghes and Beattie, 2009; Gavrilina et al., 2008). There is evidence that motor neurons require higher levels of SMN compared to other cells in order to function. When SMN levels are mildly lowered, motor neurons are the predominantly affected cells. Only when SMN levels are severely lowered do other cell types also become impaired (Burghes and Beattie, 2009; Gavrilina et al., 2008).

The question remains whether ALS resulting from defects in RNA biology shares a common downstream mechanism with ALS for which there is yet no clear link with RNA biology, such as SOD1-ALS.

1.1.4.2. Disruptions in Autophagy

Another common mechanism that involves multiple ALS-linked genetic factors is autophagy. Autophagy is a mechanism critical to cellular homeostasis and is involved in normal recycling of
organelles and certain proteins through lysosomal degradation or, clearance of dysfunctional organelles or sequestered misfolded protein inclusions. Controlled levels of autophagy are important for normal cellular function. Increased levels of autophagy can promote cell death, while decreased levels can lead to accumulation of protein aggregates and organelle material that should normally be turned over, which can lead to cellular dysfunction (Majcher et al., 2015). Many ALS-linked genes have roles associated with autophagy. These include autophagy regulators valosin-containing protein (VCP) and tank binding protein kinase (TBK1), and receptors optineurin (OPTN), ubiquilin 2 (UBQLN2), and sequestosome 1 (SQSTM1, encodes p62 protein) (Majcher et al., 2015). In addition, hexanucleotide repeat expansions in C9orf72, which have been linked to ALS, could impair autophagy (Sellier et al., 2016; Sullivan et al., 2016). It remains to be determined whether enhancing or inhibiting autophagy would be therapeutically relevant in the context of motor neuron degeneration in ALS.

1.1.4.3. ALS As a Proteinopathy

A mechanism that involves common neuropathology, but not common genetics, is the notion that the inclusions found in motor neurons in ALS indicate that the disease, at least in part, is a proteinopathy. The propensity of mutated forms of SOD1 to misfold and the fact that wild-type SOD1 forms stable dimers, led early studies to investigate the notion that accumulation of misfolded mutant SOD1 could prime wild-type SOD1 to misfold as well, thus propagating the disease process (Bruijn et al., 1998; Kabashi et al., 2007). While this is not a proven mechanism of ALS, TDP-43 inclusions have also recently been suggested to have prion-like spread (Nonaka et al., 2013). The fact that motor neurons in ALS tissue contain inclusion bodies suggests that they
are either a consequence or a cause of dysfunction, or both. For example, the sequestration of key proteins into inclusions, due to defects in trafficking, autophagy, etc., could lead to further loss of important cellular functions.

1.1.5. Links Between Observed Cellular Dysfunctions in ALS and Disease Mechanism

1.1.5.1. Endoplasmic Reticulum (ER) Stress

ER stress is a stress response in which misfolded proteins accumulate in the ER lumen, leading to activation of the Unfolded Protein Response (UPR). UPR activity is sustained until ER homeostasis is restored by the clearance of misfolded proteins from the ER lumen. However, sustained activation of ER stress can lead to apoptosis. It has been suggested that misfolded proteins such as mutated SOD1 can accumulate in the ER of motor neurons leading to sustained ER stress and eventual apoptosis. Indeed, in the mutant SOD1 mouse model of familial ALS, accumulation of misfolded SOD1 and increased ER stress-related genes in motor neurons has been reported (Kikuchi et al., 2006; Saxena et al., 2009).

1.1.5.2. Mitochondrial Dysfunction

Normal transport and function of mitochondria are essential for neuronal survival. Anterograde transportation and retrograde transport of the mitochondrial network to meet regional energetic demands are especially essential for motor neurons, which have some of the longest axons in the body. Experimental evidence in models of ALS has demonstrated a variety of defects in mitochondria that could contribute to the neurodegenerative pathology of motor neurons, including mitochondrial transport, bioenergetics, calcium buffering, and induction of mitochondrial-
mediated apoptosis (reviewed by Cozzolino et al., 2013). Magrané, et al. (2014) showed defects in mitochondrial transport in both SOD1- and TDP43-mutant mice that correlated with onset and progression of motor abnormalities (Magrané et al., 2014).

1.1.5.3. Differential Degeneration Susceptibility of Motor Neurons in ALS

Motor neurons are differentially susceptible to degeneration in ALS. For example, oculomotor neurons are spared in the disease and have been the focus of studies examining genetic and functional properties involved in differential susceptibility (Kaplan et al., 2014). Motor neurons can be subcategorized in a number of ways, one being their participation in motor units. Spinal motor neurons can be separated into three subtypes: alpha, innervating extrafusal muscle fibers; beta, and gamma motor neurons, innervating intrafusal muscle fibers. Alpha motor neurons are distinctly susceptible in ALS. The motor units innervated by alpha motor neurons can further be classified into three functional subtypes: fast-twitch fatigable (FF), fast-twitch fatigue-resistant (FR), and slow-twitch (S) (Burke and Tsairis, 1973). These motor units are characterized based on two properties: amount of force generated by the muscle, and fatigability. FF motor units generate the most force, but over a brief period of time as they fatigue quickly, whereas S motor units are required for sustained low-force movements and are more resistant to fatigue, and FR exhibit an intermediate ability to generate force and sustain contractions. Motor neurons participating in FF motor units, innervated by large alpha motor neurons, show the earliest signs of degeneration followed by FR. In contrast, S motor neurons, innervated by relatively smaller alpha motor neurons, are largely spared until the disease has significantly progressed (Fischer et al., 2004; Hegedus et al., 2007; Pun et al., 2006).
1.1.5.4. Motor Circuit Dysfunction in ALS

One hypothesis for why distinct pools of motor neurons are selectively susceptible in ALS (Kanning et al., 2010) is through their inherent differences in electrophysiologic properties and connectivity within the motor circuit. Consequently, one of the alterations observed in ALS models is a change in electrophysiological properties of motor neurons correlating with disease progression (reviewed by Leroy and Zytnicki, 2015; Quinlan, 2011). So far, defects in the motor circuit have not been demonstrated to contribute to the degenerative process in motor neurons per se. In fact, rather compelling evidence in a mouse model of SMA would argue that failure of the circuit and the actual death process of motor neurons are two distinct mechanisms (Fletcher et al., 2017).

1.1.5.5 Axonal Retraction from Nerve Terminals and Motor Neuron Death in the Spinal Cord Occur via Distinct Mechanisms

Motor neuron degeneration in ALS occurs by retraction of axons from the neuromuscular junction and axonal degeneration followed by loss of the motor neuron cell bodies residing in the spinal cord. These two events are temporally isolated, in that retraction precedes motor neuron cell death that is re-capitulated in the SOD1 mouse model of ALS, which are discussed in section 1.2.1. One demonstration that these two events occur via distinct mechanisms is that the homozygous germline deletion of Bcl-2-associated X protein (BAX), encoding a proapoptotic protein, fully rescues cell bodies of motor neurons but fails to rescue axonal degeneration in the SOD1 mouse model of ALS (Gould et al., 2006). If these two processes indeed occur via distinct molecular
events, the approach for therapeutic targeting of ALS pathogenesis will need to be adjusted. Therapies may need to be combinatorial in order to target both mechanisms, unless a common targetable molecular event is identified. Furthermore, given that motor circuit dysfunction also contributes to the disease as well as the degenerative process of motor neurons, even therapies targeted at preserving axons and cell bodies of motor neurons may not be sufficient to restore fully functional motor circuits.

1.1.5.6. Interplay Between Proposed Mechanisms of Motor Neuron Degeneration

None of these proposed mechanisms of cellular dysfunction in ALS are mutually exclusive. For example, C9orf72 mutations lead to expansions of a hexanucleotide repeat. The RNA resulting from this repeat has been shown to lead to intranuclear RNA foci that could disrupt the normal production of important proteins by impairing mRNA splicing and transport from the nucleus. This, in turn, could lead to dysfunction in any of the proposed pathways in which those sequestered proteins are normally involved. On the other hand, the C9orf72 intronic repeat expansion could also lead to haploinsufficiency of C9orf72, which may be inadequate for proper autophagy initiation. Therefore, haploinsufficiency of C9orf72 and defects in autophagy could lead to impaired clearance of ubiquitinated inclusions, which could damage the cell by sequestering critical proteins.

1.1.5.7. Non-Cell Autonomous Mechanisms in ALS

It is clear that many altered cellular pathways in ALS could lead to dysfunction within motor neurons and downstream degeneration. However, over the last 20 years, there has been a growing
body of evidence that not only do motor neuron cell-autonomous mechanisms contribute to the disease, but also non-cell-autonomous mechanisms. Toxicity to motor neurons conferred by non-neuronal neighboring cells, such as from neighboring glia, e.g., astrocytes and microglia (Frakes et al., 2014; Haidet-Phillips et al., 2011; Nagai et al., 2007; Papadeas et al., 2011; Re et al., 2014; Tong et al., 2013) correlate with disease progression. As far as neuronal cells are concerned, defects in the motor circuit conferred by dysfunction in the recurrent inhibitory circuit with interneurons (Wootz et al., 2013), also can contribute to motor circuit dysfunction, but, as discussed in section 1.1.5.4., it is unclear whether this can directly drive the degenerative process.

1.1.6. Astrocyte-Mediated Motor Neuron Cell Death

Studies in chimeric and conditional mutant SOD1-expressing transgenic mice support the notion that mutated SOD1 (mSOD1), in both motor neurons and non-neuronal cells, and especially in glial cells, contribute to the disease process in vivo (Papadeas et al., 2011; Yamanaka et al., 2008). Consistent with this evidence of non-cell-autonomous effects of mSOD1, previous work utilizing a neuron/glial co-culture model in the Przedborski lab and by others have demonstrated that astrocytes expressing mSOD1 or media conditioned by these astrocytes trigger the selective death of primary spinal motor neurons, regardless of whether the motor neurons express mSOD1 (Di Giorgio et al., 2007; Nagai et al., 2007). Evidence suggests the selective death of motor neurons in response to mutant astrocytes in vitro is due to release of a toxic factor rather than the lack of beneficial effect (Nagai et al., 2007). Recent in vitro and in vivo studies also suggest that the contribution of astrocyte non-cell-autonomous toxicity to motor neurons is a mechanism also in sporadic ALS (Haidet-Phillips et al., 2011; Qian et al., 2017; Re et al., 2014; Tong et al., 2013).
My work in this thesis focuses specifically on a mechanism of motor neuron cell death, namely necroptosis. Necroptosis was first identified as downstream to non-cell-autonomous toxicity by astrocytes associated with familial and sporadic ALS in co-culture models, which are discussed in Chapter 2. Whether necroptosis contributes to or synergizes with proposed mechanisms of cell-autonomous mechanisms of motor neuron dysfunction preceding the degeneration are also discussed.

1.2. Rodent Models of ALS

Although much recent work has been carried out to generate new rodent models based on newly-identified ALS-causing mutations in recent years, I discuss with emphasis only those mouse models relevant to the series of studies I conducted in this thesis, namely those related to SOD1 and optineurin (OPTN). Indeed, of the attempts to generate rodent models that recapitulate the hallmarks of the ALS phenotype—namely the retraction of nerve terminals from the neuromuscular junction, motor neuron cell death, progressive paralysis and premature fatality—few have succeeded in mimicking the severe pathophysiology of the human disease.

1.2.1 Mouse Models of SOD1 Mutations

The best and most enduring mouse models for the study of motor neuron cell death, mimicking the onset, progression, and severity of the human disease, are in those that grossly overexpress (15-20 copies) a human mutated SOD1 transgene with a guanine to alanine substitution at position 93, abbreviated here as SOD1\textsuperscript{G93A} (Gurney et al., 1994). These mice exhibit 40-50\% motor neuron
loss in the lumbar spinal cord by end stage, preceded by extensive retraction of nerve terminals from the neuromuscular junction, especially those originating in lumbar L4 and L5 of the spinal cord, such as the tibialis anterior muscle. In contrast to the human disease, these mice do not mimic the extent of the disease along the corticospinal tract, as pathology is primarily limited to lower motor neuron involvement.

The SOD1 gene encodes a ubiquitously expressed cytosolic protein superoxide dismutase-1, an enzyme that neutralizes harmful superoxide radicals. In fact, over 150 mutations in SOD1 have been linked to ALS, and interestingly can occur throughout the length of the protein, rather than being concentrated in a specific functional domain. The G93A mutation is not common and found in only a small number of families, but is important to the understanding of ALS in that SOD1 retains its known enzymatic activity. Therefore, the mouse model suggests that, in combination with the fact that mice lacking SOD1 do not develop motor neuron disease (Reaume et al., 1996), the role of SOD1 in ALS is less likely to be a loss of normal enzymatic function, but rather a gain of toxic function or property of the protein. Proposed mechanisms center around the propensity of mSOD1 to misfold and gain one or more toxic properties as a result, including catalyzing aberrant reactions or, as has been observed in ALS tissue, forming aggregates that associate with cytoplasmic inclusions in motor neurons as previously discussed in section 1.1.4.3. The toxicity of such aggregates is unknown, but there is some evidence of prion-like proteinopathy as a mechanism of SOD1 gain of function toxicity in ALS (Ayers et al., 2016; Lee and Kim, 2015).

Other mouse models of SOD1 mutations lead to similar phenotypes, with varying lifespans, and depend greatly on transgene copy number and the background strain in which it is generated. The SOD1<sup>G93A</sup> transgenic mouse has been the subject of criticism, mainly because of the extreme
overexpression (15-20 copies) needed to recapitulate the disease, and the dearth of experimental findings in the model that have translated therapeutically to the human condition (Zwiegers and Shaw, 2015). Despite this, the SOD1\textsuperscript{G93A} transgenic mouse remains one of the most well-characterized and widely-used mouse models. In addition, for the purposes of my work, which is focused on motor neuron cell death, it was imperative that the mouse model recapitulated this aspect of the pathology of the disease.

Importantly, SOD1\textsuperscript{G93A} transgenic mice have variable onset and end stage based on the background strain used and copy number of the transgene. When the transgene is expressed in a mixed background B6/SJL, the average lifespan is about 4 months, whereas when expressed in the C57Bl/6J background, it is about 5 months. Sex differences in survival have also been reported for the B6/SJL background, with female mice living slightly longer and with a later onset of ALS-like motor manifestations (Heiman-Patterson et al., 2005; Scott et al., 2008).

1.2.1. Mouse Models of Optineurin Deficiency

Recently, optineurin (OPTN) mutations, first identified in glaucoma, have been linked to familial ALS (Maruyama et al., 2010). Many of these mutations correlated with loss of function and haploinsufficiency; thus, it has been suggested that optineurin plays a neuroprotective role (reviewed by Markovinovic et al., 2017). In part, in order to dissect the relevance of OPTN loss to ALS, Ito et al. (2016) generated a homozygous germline deletion of optineurin (OPTN\textsuperscript{-/-}) and found a very mild phenotype with some evidence of axon degeneration in the white matter of the spinal cord. This was attributed to non-cell-autonomous contributions of increased microglia

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activation and white matter oligodendrocyte degeneration, having a mild degenerative effect on myelinated axons in the ventral spinal cord white matter.

1.3. Exploring Molecular Determinants of Apoptosis in Experimental Mouse Models of ALS and In Vitro Models

The downstream execution mechanisms owing to eventual neuronal demise in neurodegenerative diseases continue to be an area of open investigation. Motor neuron retraction from nerve terminals and cell death in ALS occur throughout the entire spinal cord and in the upper motor neurons of the cortex, while other cells and neuronal processes in the spinal cord and in the cortex, besides notably gray matter oligodendrocytes (Kang et al., 2013), remain largely intact. Regardless of the interesting areas of exploration regarding the initiators of this death, one important area to be explored for clinical intervention is the downstream effectors, e.g., the molecular mechanisms, if any, which execute motor neuron death.

1.3.1. Targeting Apoptosis in Mouse Models of ALS

1.3.1.1. Overexpression of Bcl-2

Bcl-2 (B-cell lymphoma 2) is a negative regulator of proapoptotic proteins, and its overexpression has been shown to inhibit apoptosis. Kostic et al. (1997) overexpressed Bcl-2 in the nervous system including motor neurons in SOD1<sup>G93A</sup> mice and found a delay in onset of
Figure 1.6. Targeting Apoptosis in the SOD1$^{G93A}$ Mouse Models of ALS

**a.** Kostic et al. (1997) overexpressed Bcl-2 in the nervous system, including motor neurons, in SOD1$^{G93A}$ mice and measured onset of motor symptoms (top) and survival (bottom). (circle=control, triangle=Bcl-2 overexpression)

**b.** Li et al. (2000) used osmotic pumps to deliver zVAD-fmk to the cerebral ventricle of SOD1$^{G93A}$ mice and measured onset of motor symptoms (top) and survival (bottom). (dashed=vehicle, line=zVAD-fmk)

**c-e.** Gould et al. (2006) generated SOD1$^{G93A}$ mice that were homozygous for the germline deletion of the proapoptotic protein Bax and measured onset of motor symptoms (c), survival (d), and lumbar spinal motor neuron number (e) at end stage.
motor symptoms in mice that correlated with a shift in overall survival of the mice and an improvement in the number of surviving motor neurons at end stage (Figure 1.6, a). However, time from onset to death, which one could think of as a measure of progression, was not changed (Figure 1.6, a) (Kostic et al., 1997).

1.3.1.2. Pharmacological Targeting of Caspases

Li et al. (2000) used osmotic pumps to deliver N-benzyloxy carbonyl-Val-Asp-fluoromethylketone (zVAD.fmK) to the cerebral ventricle of SOD1<sup>G93A</sup> mice. ZVAD.fmK is a broad-spectrum inhibitor of caspases (Garcia-Calvo et al., 1998) whose use, in many models, is sufficient to inhibit apoptosis. Like overexpression of Bcl-2, there was a delay in onset of motor symptoms which correlated with an overall survival of the mice and better performance of the mice on motor ability tests. However, like in the overexpression of Bcl-2, time from onset to death was not changed (Li, 2000) (Figure 1.6, b).

1.3.1.3. Homozygous Germline Deletion of Bax

Perhaps the most compelling evidence of the contribution of programmed cell death in SOD1<sup>G93A</sup> mice came from the studies of Gould et al. (2006) who generated SOD1<sup>G93A</sup> mice that were homozygous for the germline deletion of the gene encoding the proapoptotic protein Bax (Bax<sup>−/−</sup> mice). Bax<sup>−/−</sup> mice have an increased number of motor neurons, owing to the critical role of Bax in developmental motor neuron death (White et al., 1998). However, Bax<sup>−/−</sup>/SOD1<sup>G93A</sup> completely rescued motor neuron death even at end stage, having the same increased number of motor neurons as non-transgenic Bax<sup>−/−</sup> mice (Figure 1.6, e). Interestingly, although motor neuron number in the
lumbar spinal cord was completely rescued, there was no improvement in motor axonal degeneration or retraction from the neuromuscular junction, which, at least in part, supports the idea that these two events are mechanistically distinct in ALS as discussed in section 1.1.5.5. As in the studies of Li et al. (2000) and Kostic et al. (1997), there were very modest improvements in onset and survival in these mice (Figure 1.6, c-d), likely attributed to the lack of ability to mitigate axonal degeneration.

There were very modest effects of targeting apoptosis in mouse models of ALS, except in the case of Bax. Indeed, Bax is critical for motor neuron death in response to the non-cell-autonomous effects of familial and sporadic ALS astrocytes (Nagai et al., 2007; Re et al., 2014), which we recently attributed to a mechanism of nonapoptotic cell death (Re et al., 2014). This and the lack of apoptosis targeting to completely mitigate the disease in mice further suggests that caspase-mediated cell death does not contribute to the full extent of motor neuron death in the disease. Despite some evidence of caspase activation in ALS mouse models and even in human tissue (Inoue et al., 2003; Li, 2000; Pasinelli et al., 2000; Vukosavic et al., 2000), others have reported an inability to detect clear signs of apoptosis in the degenerating motor neurons in the mouse models (Migheli et al., 1999). More recently, as insights into characterization of programmed cell death (PCD) mechanisms have been gained, and a variety of nonapoptotic programmed cell death mechanisms have emerged (Table 1.2, reproduced from Vanden Berghe et al., 2015) which challenge the historical dichotomy between apoptosis and necrosis. One such mechanism is necroptosis, which are discussed in section 1.4.
1.4. Necroptosis is an Alternative Programmed Cell Death Pathway

Necroptosis has emerged as an alternative form of PCD in a variety of disease contexts (Ofengeim et al., 2015; Weinlich et al., 2016). Necroptosis is a form of programmed cell death with distinct molecular regulation from that of apoptosis, most strikingly the independence from caspase activation. Cells display features of necrosis rather than apoptosis, such as early loss of plasma membrane integrity and swelling (oncosis). Cell death by necroptosis in vivo induces inflammation in tissues much like necrosis through membrane rupture and exposure of cellular components to the immune system (Vanden Berghe et al., 2015).
1.4.1 Molecular Characterization of Necroptosis

Activation of necroptosis first occurs at the plasma membrane by ligation of a death receptor or toll-like receptor, canonically tumor necrosis factor receptor-1 (TNFR1) by tumor necrosis factor (TNF), but many other ligand-receptor interactions to activate necroptosis have been described (Vanden Berghe et al., 2016).

Activation of TNFR1 can lead to a variety of downstream signaling, including induction of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-dependent transcription of proinflammatory and pro-survival genes, via formation of Complex I, FAS-associated via death domain (FADD)/caspase-8 mediated apoptosis, via formation of Complex II, and necroptosis signaling, via formation of the necrosome (Figure 1.7.) (Pasparakis and Vandenabeele, 2015).

Necroptosis was first described using in vitro models as an alternative cell death to apoptosis when cells were treated with TNF and apoptosis was concomitantly inhibited using the pan-caspase inhibitor zVAD.fmk. It was first determined that the kinase activity of receptor-interacting protein kinase-1 (RIPK1) is essential for necroptosis, as the discovery that necrostatin-1, a potent allosteric inhibitor of RIPK1 kinase function, fully inhibits TNF/zVAD.fmk-induced necroptosis (Degterev et al., 2005, 2008). Subsequent biochemical in vitro studies identified that RIPK1 interacts with receptor-interacting protein kinase-3 (RIPK3) in a phosphorylation-dependent manner, forming a stable complex via homotypic interactions in their RHIM domains (e.g., “necrosome”) necessary for necroptosis (Cho et al., 2009; He et al., 2009; Zhang et al., 2009). RIPK3 then acts as an adapter that recruits and phosphorylates downstream targets to the necrosome, notably the pseudokinase mixed lineage kinase domain-like-pseudokinase (MLKL) (Sun et al., 2012; Zhao et al., 2012). More recently, it has been shown that the execution of
necroptosis depends on MLKL translocation to the plasma membrane and assembly into oligomeric membrane-permeable pores (Cai et al., 2014; Chen et al., 2014; Su et al., 2014; Wang et al., 2014) with striking structural similarity to the pores formed by Bax on the mitochondrial outer membrane during apoptosis.

1.4.2 Evidence for Roles of Necroptosis in Human Disease

Since the characterization of necroptosis in vitro induced by stimulation with TNF and inhibition of necroptosis with zVAD.fmk, the contribution of necroptosis has been implicated in a number of cellular contexts and disease models. The most well-characterized role of necroptosis is in the field of immunology. One proposed evolutionary basis for necroptosis is that it acts as a defensive cell death mechanism against viruses that express anti-apoptotic proteins, with the resulting inflammation further propagating an immunological response (Newton and Manning, 2016). Studies using RIPK3 knockout mice have demonstrated the role of necroptosis in the cellular response to microbial infections, as well as in disease models that involve inflammation-induced tissue damage (Moriwaki and Chan, 2013; Newton, 2015). Disease models ameliorated by the genetic targeting of necroptosis are listed in Table 1.3 reproduced from Newton (2015).
Recently, in a study I participated in using in vitro models, necroptosis was implicated as the mechanism in ALS by which ALS astrocytes may confer non-cell-autonomous cell death to motor neurons (Re et al., 2014) (described in more detail in Chapter 2). More recently, Ito et al. (2016) described an alternative mechanism for the role of necroptosis in ALS, again in a non-cell-autonomous mechanism in which necroptosis activation in microglia leads to oligodendrocyte loss, which leads to motor neuron myelination defects and the resulting axonopathy (Ito et al., 2016a), a study discussed further in Chapter 5 as it relates to the present work.
Table 1.3. Genetic Targeting of Necroptosis in Various Disease Models

<table>
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<tr>
<th>Disease models ameliorated in mice expressing catalytically inactive RIPK1 (RIPK1&lt;sup&gt;kd/kd&lt;/sup&gt;) or lacking RIPK3 (RIPK3&lt;sup&gt;-/-&lt;/sup&gt;)</th>
<th>Tested in</th>
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<tr>
<td>Model</td>
<td>RIPK3&lt;sup&gt;-/-&lt;/sup&gt;</td>
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<td>Skin and multi-organ inflammation in Sharpin mutant mice</td>
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<td>Systemic inflammation induced by TNF</td>
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<td>Cerulein-induced pancreatitis</td>
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<td>Atherosclerosis in Ldr or Apoe mutant mice</td>
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<td>dsRNA-induced retinal degeneration</td>
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<td>rd10 model of retinitis pigmentosa</td>
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<td>Kidney ischemia-reperfusion injury</td>
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<td>Gaucher's disease</td>
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<td>Ethanol-induced liver injury</td>
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1.5. Specific Aims

The overall goal of my studies has been to shed light on the role of necroptosis in a neurodegenerative disorder such as ALS and to assess whether necroptosis, if instrumental here, operates within motor neurons via the same core machinery as in other cell types, especially immune cells.

To achieve these stated goals, my thesis comprises four experimental Chapters, namely Chapter 2 to 5:

In Chapter 2, I establish the role of necroptosis as the mechanism of astrocyte-mediated motor neuron death in in vitro models of familial and sporadic ALS. I show that while motor neurons engage core components of necroptosis, a number of differences emerge between motor
neuron necroptosis and what has been reported in the literature. In addition, there is increasing evidence in the literature that cell death pathways are cell context specific.

Building on these findings, in Chapter 3, I investigate the motor neuron-specific cell death machinery, which we now know is driven by core elements of necroptosis, by meta-analyzing a gene expression signature captured from motor neurons undergoing cell death that was previously generated in the lab by Ikiz et al. (2015).

In Chapter 4, I explore the in vivo relevance of necroptosis in mouse models of ALS, by first validating immunoreagents that are mandatory for allowing the study of the core elements of necroptosis in mouse tissue, namely RIPK1, RIPK3, MLKL and phosphorylated MLKL. While I was able to identify several reliable immunoreagents for Western blotting of whole tissues, thus far I have not been able to identify immunoreagents that could detect necroptosis components at the single-cell level in mouse spinal cord using immunohistochemistry.

Lastly in Chapter 5, I seek to more directly and mechanistically assess the contribution of necroptosis to the disease phenotype in a mouse model of ALS by deleting the RIPK3 gene in transgenic SOD1$^{G93A}$ mice.

My overall conclusion of the work in Chapters 2-5 is that while the necroptosis machinery drives motor neuron death in in vitro models of ALS, more work needs to be done to (1) assess the motor neuron-specific cell death program, and (2) evaluate the relationship, if any, of necroptosis to motor neuron disease in vivo.
Chapter 2: Programed Cell Death and Motor Neuron Degeneration

2.1. Introduction

The observation that motor neurons die in response to co-culture with SOD1\textsuperscript{G93A}-expressing astrocytes was documented previously in the Przedborski group and by others interested in non-cell-autonomous mechanisms of neurodegeneration in ALS (Di Giorgio et al., 2007; Nagai et al., 2007). To describe briefly the co-culture model, primary motor neurons (PMNs) are co-cultured with primary neonatal astrocytes from rodents overexpressing the human mutant transgene SOD1\textsuperscript{G93A}, a rodent model of familial ALS (fALS) that recapitulates most hallmarks of ALS (Gurney et al., 1994).

It was reported that after 7 days in culture, 50% of motor neurons selectively die in response to co-culture with SOD1\textsuperscript{G93A}–expressing astrocytes compared to wild-type astrocytes (Figure 2.1 a, b). In contrast, other neurons, such as spinal GABAergic, interneurons, or dorsal root ganglion neurons, showed no difference in survival in culture with SOD1\textsuperscript{G93A}–expressing astrocytes (Figure 2.1 a, b) (Nagai et al., 2007). These observations supported the claim that this in vitro model not only recapitulated a spontaneous neurodegenerative phenotype caused by a human ALS-relevant context, i.e., by mutated SOD1 (mSOD1), but also that it reproduced the characteristic feature of ALS that not every population of spinal neurons were equally susceptible to the disease process (Kanning et al., 2010).

The Przedborski lab also provided evidence that this motor neuron death was not due to a loss of beneficial properties from the mutant astrocytes, but rather a deleterious gain of function.
related to the release of a toxic factor by the mutant astrocytes. Indeed, it was demonstrated that medium conditioned with mutant but not wild-type astrocytes and re-supplemented with growth factors and glucose conferred the same toxicity as co-culture (Nagai et al., 2007).

Figure 2.1. Establishment of the fALS Co-culture Model

Figure reproduced from Nagai et al. (2007) Nat. Neurosci.

a. Wild-type primary motor neurons isolated from E12.5 embryos, expressing GFP under the HB9 motor neuron-specific promoter, were co-cultured on primary astrocyte monolayers from SOD1G93A or non-transgenic (NTg) rats. The percentage of surviving primary motor neurons (identified by immunostaining for GFP) on SOD1G93A at 7 days was lower (p<0.004) than at 1 day, whereas the number of surviving GABAergic neurons was identical at 1 day and 7 days (P > 0.5) b. Mouse embryonic stem cell-derived cultures, containing motor neurons expressing GFP under the HB9 motor neuron-specific promoter, Lim1/2+ neurons negative for GFP (likely interneurons) and other neurons positive for pan-neuronal marker MAP2+ but not GFP, were plated on primary astrocyte monolayers from SOD1G93A or non-transgenic (NTg) rats. At five days in co-culture, the percentage of surviving motor neurons on SOD1G93A astrocytes was lower (P<0.01) than on NTg astrocytes, whereas the percentage of the Lim1/2+ interneurons (p=0.9) was not changed nor was the percentage of other neurons (P=0.8).
After the in vitro model of fALS was established, efforts were made to observe and characterize the mode of motor neuron cell death in response to mutant astrocytes, which indeed could be very different from cell-autonomous mechanisms that had been thus proposed downstream of endoplasmic reticulum stress (Kikuchi et al., 2006; Saxena et al., 2009) and mitochondrial dysfunction (Magrane et al., 2009, 2011). One of the first distinctions to make was whether motor neurons were dying via a molecularly-controlled mechanism, the most prevalent being classical apoptosis, or by a passive mechanism via necrosis. Signs that this was a molecularly-controlled event were that motor neurons undergoing cell death in this in vitro model displayed increased DNA fragmentation, a hallmark of PCD, observed by TUNEL positive staining, and that motor neurons had increased caspase-3 activation, a hallmark of apoptosis, observed by immunostaining for fractin, a cleavage product produced by activated caspase-3 during apoptosis (Fig 2.2, a) (Nagai et al., 2007). In addition, primary motor neurons cultured from mice with homozygous germline deletion for Bax (Bcl-2-associated X), a protein with known function in PCD execution, were resistant to death in response to SOD1G93A-expressing astrocytes (Fig. 2.2, b).

In light of these data, I embarked on the characterization of the form of PCD that drives the demise of motor neurons in response to mutant astrocytes. In this chapter, I provide evidence that motor neurons selectively die in co-culture with ALS-associated astrocytes in a molecularly-regulated manner by the necroptosis pathway. Understanding the mechanism by which motor neurons die in ALS is of great importance to understanding the neurodegeneration that contributes to the disease pathology as well as identifying molecular targets for intervention.
a. Mouse embryonic stem cell-derived motor neurons (ES-MNs) were plated on primary astrocyte monolayers from SOD1^{G93A} mice or non-transgenic (NTg) rats for 7 days. **top:** All ES-MNs immunopositive for fractin (red) also showed DNA condensation (blue), as evidenced by Hoechst 33342 **bottom:** all ES-MNs with Hoechst 33342–labeled chromatin clumps show DNA fragmentation, as evidenced by TUNEL. Scale bar, 20 μm. TUNEL=terminal dUTP nick-end labeling. 

b. Wild-type or Bax knockout primary motor neurons isolated from E12.5 mouse embryos, were co-cultured on primary astrocyte monolayers from SOD1^{G93A} or non-transgenic (NTg) mice for 7 days and surviving motor neurons were counted using SMI32+ immunolabeling to identify motor neurons. A two-way ANOVA revealed there is an interaction between the astrocyte genotype and the motor neuron genotype (p<0.001). Newman-Keuls post-hoc analyses indicated there are fewer (p<0.001) wild-type motor neurons whereas there was no difference (p=0.939) in the number of surviving Bax KO motor neurons on SOD1^{G93A} monolayers. Values express MN survival in percent of controls (i.e. MNs number cultured on NTg astrocytes) and represent means ± SEM (n=3-4 per group). Bax KO= motor neurons from mice with homozygous germline deletion of Bax.

### 2.2. Results

Over the past decade, a host of new forms of PCD have emerged, often linked to specific cell death paradigms and often with a high degree of cell-type specificity (Feoktistova and Leverkus, 2015; Fuchs and Steller, 2015). Thus, even if apoptosis is the most prevalent form of PCD, it is essential...
to consider alternative forms of molecularly-controlled mechanisms of motor neuron degeneration. This view is particularly relevant to mSOD1-related motor neuron degeneration in ALS transgenic mice, in which morphological features of apoptosis are rather rare and targeting apoptosis in transgenic mSOD1 mice by inhibiting caspases has provided limited benefit (discussed in Chapter 1, section 1.3).

To begin my characterization of PCD in this in vitro model of ALS, I revisited the role of caspases, a family of cysteine proteases, which play key regulatory and execution roles in all forms of apoptosis (Budihardjo et al., 1999). Consistent with previous finding of this lab (Nagai et al., 2007; Re et al., 2014), I found that when cells were treated with pan-caspase inhibitor zVAD-fmk (Nagai et al., 2007) as well as with inhibitors targeting individual caspases such as Ac-DNLD-CHO for caspase 3/7 or zIETD for caspase-8, the magnitude of motor neuron loss between the cultures containing mutant or wild-type astrocytes did not differ (Fig 2.3, a).

In striking contrast, while survival of motor neurons was not afforded by zVAD-fmk, this treatment did abrogate all features of apoptosis, such as fraction-positive cells in motor neurons incubated with mutant astrocyte-conditioned medium (Nagai et al., 2007). Cultured motor neurons were also not protected from mutant astrocytes using soluble Fas ligand antagonist (Nagai et al., 2007), ruling out a Fas-dependent apoptosis mechanism of motor neuron cell death, which had been previously described (Raoul et al., 2002).
However, early evidence pointed toward a role of a nonapoptotic mechanism of PCD in motor neurons, in that motor neurons in co-culture with SOD1G93A-expressing astrocytes showed early loss of plasma membrane integrity, imaged by ethidium homodimer permeability assay, a feature of necrosis rather than apoptosis (Re et al., 2014). In apoptosis, plasma membrane integrity is lost much later in the cell death process rather than occurring as a primary event. Thus, the story unfolded of a caspase-independent motor neuron cell death mechanism with morphological features reminiscent of necrosis. In light of this, I sought to examine the role of oxygen and nitrogen reactive species by using SOD1 mimetics (Jung et al., 2001), N-acetylcysteine (Ferrari et al., 1995) or nitric oxide synthase inhibitors (Moore and Handy, 1997; Watanabe et al., 2004), and none provided any protection. Likewise, PARP-1 inhibitors (Fatokun et al., 2013), ferostatin-
1 (Dixon et al., 2012) or AMPA/Ka receptor antagonists (Nagai et al., 2007; Van et al., 2003) aimed at preventing ferroptosis, parthanatosis or excitotoxicity, respectively, did not attenuate the loss of motor neurons in response to mutant astrocyte-conditioned medium.

However, when necrostatin-1 (Nec-1) was used there was near complete protection of motor neuron death (Re et al., 2014) (Figure 2.4). These results strongly argue for the death of motor neurons in response to mutant astrocytes being driven by necroptosis, a form of molecularly-regulated necrosis introduced in detail in Chapter 1.

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**Figure 2.4. Astrocyte-mediated Motor Neuron Death is Rescued by Inhibiting RIPK1 Kinase Function**

Wild-type primary motor neurons (MN) isolated from E12.5 embryos, expressing GFP under the HB9 motor neuron-specific promoter, were co-cultured on primary astrocyte monolayers from SOD1<sup>G93A</sup> or non-transgenic (NTg) mice. The percentage of surviving primary motor neurons on SOD1<sup>G93A</sup> at 7 days compared to NTg was measured (identified by immunostaining for GFP). While vehicle (Veh) showed significant motor neuron loss (DMSO, p<0.001), the RIPK1 inhibitor necrostatin-1 (Nec1, 5 µM) abrogated the MN loss (p=0.532). DMSO = dimethylsulfoxide.

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As indicated in Chapter 1, known core components of necroptosis include RIPK1, RIPK3 and MLKL. Briefly, necroptosis signaling occurs downstream of ligand-mediated death receptor activation at the plasma membrane, leading to phosphorylation-dependent assembly of a key molecular complex containing RIPK1 and RIPK3, termed the necrosome (Cho et al., 2009; He et al., 2009; Zhang et al., 2009). This allows RIPK3 to form amyloid-like signaling oligomers (Li et al.), and recruit and phosphorylate downstream targets to execute cell death, most notably the pseudokinase MLKL, a protein with strikingly similarity in structure to Bax, which has been
shown to have oligomeric pore-forming ability at the plasma membrane (Cai et al., 2014; Chen et al., 2014; Su et al., 2014; Wang et al., 2014).

My studies thus began at this exciting time in the Przedborski group. A robust, promising in vitro model of “ALS in a dish” had suggested for the first time that necroptosis may play an important role in motor neuron cell death in ALS. If true, this would have a number of implications for both the study of necroptosis in the context of neurodegeneration and for identifying new potential therapeutic avenues for ALS.

My initial goal was to confirm targets and validate the initial findings. The small molecule drug used to inhibit cell death in our model, Nec-1, besides antagonizing the kinase function of RIPK1, also has known off-target interactions. Most notably, Nec-1 inhibits the immunomodulatory enzyme indoleamine-2,3-dioxygenase, which has known roles in innate and adaptive immunity as well as in neuroprotection (Vandenabeele et al., 2013). Therefore, protection against cell death using Nec-1 alone is not sufficient to confirm necroptosis. We first used genetic targeting of RIPK1 to confirm that the rescue observed in Nec-1 treatment was indeed due to RIPK1 involvement. When I started my project, knockdown of RIPK1 mRNA in motor neurons by shRNA-containing lentivirus had demonstrated motor neuron protection in the fALS co-culture model. To further confirm these findings, I infected cells with a lentivirus containing a second unique shRNA sequence against RIPK1, which decreased RIPK1 transcript in the motor neuron culture by 60% (Fig. 2.5, a). Knockdown of RIPK1 afforded full protection of motor neuron death on SOD1G93A-expressing astrocytes compared to motor neurons infected with non-mammalian targeting shRNA-containing control lentivirus (Fig. 2.5, b). These results demonstrated that silencing RIPK1 phenocopied the effects of Nec-1, which provided compelling evidence for the
involvement of RIPK1 in the death mechanism of motor neurons in our in vitro model of ALS.

Figure 2.5. Astrocyte-mediated Motor Neuron Death is Rescued by RIPK1 Silencing

Wild-type primary motor neurons (MN) isolated from E12.5 embryos, expressing GFP under the HB9 motor neuron-specific promoter, were co-cultured on primary astrocyte monolayers from SOD1<sup>G93A</sup> or non-transgenic (NTg) mice. Before plating, cells were infected with shRNA-containing viral particles containing an empty vector (EV), non-mammalian targeting vector (SC), or shRNA against RIPK1 by spinoculation at MOI 20 as described in (Re et al. 2014). a. After 4 days, an average of 60% reduction in endogenous RIPK1 transcript was measured by qPCR b. After 7 days, there were fewer (p<0.001) motor neurons transduced with SC on SOD1<sup>G93A</sup> astrocytes than NTg astrocytes but motor neurons with 60% reduction in RIPK1 transcript were at equal numbers at day 7 on SOD1<sup>G93A</sup> astrocytes and NTg astrocytes (p>0.1). Data represent means ± SEM (n=3) MOI=multiplicity of infection (For more detailed methods, see Experimental Procedures). Motor neurons identified by immunostaining for GFP.

Although the above results provided strong support to the idea that motor neurons in our in vitro model of ALS were dying by necroptosis, it is important to emphasize that RIPK1 is also known to operate outside of necroptosis (Festjens et al., 2007; Newton, 2015; Ofengeim and Yuan, 2013). Thus far, however, RIPK3 has no known functions outside of cell death and specifically necroptosis. Thus, to further support the hypothesis of necroptosis, I decided to then test the involvement of RIPK3. At the time of this work, unlike for RIPK1, there were no validated small
molecules available to inhibit RIPK3, and therefore, I decided to use constitutive knockout mice deficient in this kinase (Newton et al., 2004), kindly provided by Genentech. These mice develop normally to adulthood without any observed defects in weight gain or fertility, and histological analysis does not show defects in major organs (Newton et al., 2004). I thus cultured primary motor neurons from mice with homozygous germline deletion for RIPK3 (RIPK3KO) and from their wild-type counterparts. Quantitative RT-PCR showed no expression of RIPK3 mRNA transcript in the embryo at day 12.5 compared to control, in neither the head nor the spinal cord from which these cultures are derived (Fig. 2.7, a). First, I demonstrated that primary RIPK3KO spinal motor neurons have normal morphology of cell bodies and axons in culture (Fig. 2.6), because, to my knowledge, this is the first time primary neurons from these animals had been cultured. I then assessed the effect of mutant astrocytes on RIPK3-deficient motor neurons as done before in the lab for other mutant mouse lines (Nagai et al., 2007; Re et al., 2014). I found that counts of RIPK3KO motor neurons co-cultured with mutant astrocytes for 7 days did not differ from those of RIPK3KO motor neurons co-cultured with wild-type astrocytes (Student’s t-test, p=0.075) (Fig. 2.7, b). Conversely, within the same experiment, I found that counts of wild-type motor neurons co-cultured with mutant astrocytes were ~40% lower than those of wild-type motor neurons co-cultured with wild-type astrocytes (Student’s t-test, p=0.0002) (Fig. 2.7, b). These results indicated that the constitutive lack of RIPK3 also provided protection to motor neurons from the deleterious effects of SOD1<sup>G93A</sup>–expressing astrocytes. Accordingly, it seemed that the case for necroptosis, being a driver of motor neuron degeneration in this in vitro model of ALS, was becoming very strong. I could also conclude that, because both RIPK1 and RIPK3 are necessary for motor neuron death in this model of ALS, it was likely that this was a necroptosis-dependent mechanism resulting from the formation of the necrosome assembly and ensuing signaling.
RIPK3 knockout spinal motor neurons were isolated from E12.5 mouse embryos and plated on wild-type astrocyte monolayers as has been described (Nagai et al., 2007). After 7 days, SMI32 immunostaining (green) labeled motor neurons were observed with typical axonal and cell body morphology indistinguishable from wild-type motor neurons in culture prepared under comparable conditions. DAPI counterstained nuclei (blue) and MAP2 staining (red) labeled all neurons in culture.

Notably, at the same time as the non-cell-autonomous astrocyte-mediated mechanism of motor neuron death in the model of familial-associated mutation in mouse was being elucidated, a fully humanized sporadic ALS (sALS) model co-culture system was being established. For this, postmortem astrocytes from sporadic ALS patients and age-matched donor control astrocytes were co-cultured with human embryonic stem cell-derived motor neurons. I actively participated in the culture of postmortem human astrocytes from patients and controls to aid in the study of this new culture model that provided us, for the first time, the ability to test our pathogenic hypothesis in a human cell system and in absence of mutant SOD1 overexpression. Furthermore, to assure that the astrocytes were from sALS patients, each sample was genotyped and none of the known mutations
linked to ALS was identified. None of the samples used in the lab and particularly in my studies carried pathological C9orf72 expansion (Re et al., 2014). Strikingly, this fully humanized in vitro model of sALS demonstrated the same non-cell autonomous and selective motor neuron death in response to sALS-associated astrocytes (Re, 2014). Targeting of RIPK1 by shRNA-mediated knockdown or Nec-1 treatment was protective against the selective motor neuron death (Re et al., 2014). This model system also provided us with a unique opportunity to test for the involvement of MLKL at the level of its activation by using a small molecule drug called necrosulfonamide (NSA) (Sun et al., 2012). NSA operates by binding to a human-specific cysteine that is not conserved in mice and thus, the only chance to test the effect of using NSA for this work was in the humanized model that now was available to me. Accordingly, human ES-MNs were cultured with human sALS astrocytes in the presence and absence of NSA. This experiment showed that counts of human ES-MNs co-cultured with human sALS astrocytes for 7 days in the presence of 0.25 µM NSA did not differ from those of human ES-MNs co-cultured with human control astrocytes (post-ANOVA Newman-Keuls test, p>0.12) (Fig. 2.8). Conversely, within the same experiment, I found that the counts of human ES-MNs co-cultured with human sALS astrocytes in absence of NSA were ~50% lower than those of human ES-MNs co-cultured with human control astrocytes (post-ANOVA Newman-Keuls test p<0.001) (Fig. 2.8). Although these results were derived from the use of a small molecule that could have unexpected off-target effects, they suggest that MLKL is also instrumental in the demise of motor neurons. Thus, taken together, these two models of both familial and sporadic ALS suggested, for the first time, necroptosis as a generalized mechanism of motor neuron death.
Figure 2.7. RIPK3<sup>-/-</sup> motor neurons are protected from SOD1<sup>G93A</sup> astrocytes

a. Mouse E12.5 embryos from wild-type (WT) or RIPK3<sup>-/-</sup> mice (Newton et al., 2004) were dissected to remove the whole spinal cord (sc) or whole head and RNA was extracted and prepared for RT-qPCR to detect RIPK3 mRNA transcript (n=1-3 samples per condition, error bars represent SEM of biological replicates). Quantification showed relative levels of RIPK3 were undetectable in all RIP3<sup>-/-</sup> samples. 

b. Wild-type or RIPK3 knockout primary motor neurons (MN) isolated from E12.5 mouse embryos, were co-cultured on primary astrocyte monolayers from SOD1<sup>G93A</sup> or non-transgenic (NTg) mice for 7 days, and surviving motor neurons were counted using SMI32<sup>+</sup> immunolabeling to identify motor neurons. RIPK3<sup>-/-</sup> motor neurons did not differ in counts between NTg or SOD1<sup>G93A</sup> astrocytes (Student’s t-test, p=0.075), whereas WT motor neurons co-cultured with mutant astrocytes were ~40% lower on SOD1<sup>G93</sup> astrocytes (Student’s t-test, p=0.0002). Error bars represent SEM. n=3 biological replicates.
Figure 2.8. Human Astrocyte-mediated Motor Neuron Death is Protected by MLKL Inhibition

Reproduced from Re et al. (2014).

Human primary astrocytes/purified GFP+ human ES-derived motor neuron cultures were prepared as described in Re et al. (2014). In the presence of vehicle (Veh, DMSO), there were fewer (p<0.001) motor neurons on sporadic ALS patient astrocytes compared to non-diseased control astrocytes. 250 nM of the MLKL inhibitor necrosulfonamide (NSA) prevented the loss of human motor neurons co-cultured for 7 d on sporadic ALS astrocytes as hES-MN numbers no longer differed (p>0.123). Data are expressed as percent of MN number on Ctrs astro. Mean and error bars represent ± SEM (n=3–5 per group). Ctrs = astrocytes from age-matched control patients with no neurologic disease. Motor neurons identified by immunostaining for GFP.

2.3. Discussion

Our results indicated that astrocyte-mediated motor neuron death in both familial and sporadic in vitro models of ALS occured by a programmed cell death pathway that involved RIPK1, RIPK3, MLKL, and Bax, and did not involve caspases. Both inhibition of RIPK1 kinase function and silencing of RIPK1 protected motor neurons from ALS astrocyte-induced death. Motor neurons lacking RIPK3 were protected from cell death. Furthermore, in the human sporadic ALS co-culture model, motor neurons were protected from astrocyte-mediated cell death in co-culture by pharmacologic inhibition of MLKL activation. This provided strong evidence that motor neurons
respond to ALS-associated astrocyte toxicity by engaging necroptosis machinery leading to their demise. These results however raise two important questions that are explored in the following chapters:

First, while the dependence on RIPK1 kinase function and RIPK3 are clearly demonstrated, what is the mechanism of motor neuron death downstream of RIPK1/RIPK3 activation? Second, what is the dependence, if any, on necroptosis in the development of motor neuron disease and could this pathway be a good target for therapeutic intervention? I explore these questions in Chapters 3-5.
Chapter 3. Unbiased Approach to Study Motor Neuron Death in ALS

3.1. Introduction

Over the past few years, efforts have been made to decipher the molecular makeup of necroptosis, and a great deal has already been discovered, at least in the context of the immune system and in response to TNF-α and/or the pan-cascape inhibitor zVAD-fmk in cultured cells (Degterev et al., 2008; He et al., 2009; Newton and Manning, 2016). From the work done in the Przedborski lab to which I have contributed (discussed in the previous chapters), it is clear that spinal motor neurons succumb to the deleterious effects of both mouse familial (i.e., expressing mSOD1) and human sporadic ALS astrocytes via the engagement of core components of necroptosis (Re et al., 2014). However, we also discovered that while some elements were conserved, namely the dependence on RIPK1, the involvement of RIPK3, and the activity of MLKL, a number of striking differences emerged from the results. For instance, motor neuron necroptosis in the in vitro ALS models I studied in my work:

a. occurred in the absence of caspase-inhibition,

b. was dependent on the pro-cell death Bax protein,

c. was dependent on the canonical pathway of NF-κB, and

d. was equally rescued by RIPK1 inhibition and silencing.

These differences from previous publications in the literature, elaborated below, led me to hypothesize that some aspects of the necroptosis mechanism may be cell specific.
3.1.1. Necroptosis Activation in ALS.

Almost all studies that have been performed to elucidate the biochemistry and molecular mechanisms of necroptosis relied thus far on protein overexpression and immortalized cancer cell lines (Cho et al., 2009; Degterev et al., 2005, 2008). Additionally, in most of these studies, necroptosis is observed only when cell death is induced and apoptosis is concomitantly inhibited (Degterev et al., 2008; He et al., 2009; Newton and Manning, 2016). The evolutionary role for necroptosis as an alternative death pathway of apoptosis when the latter is inhibited is proposed as a mechanism related to innate immunity. Indeed, some pathogens such as viruses and bacteria express anti-apoptotic proteins, hence preventing the engagement of apoptosis (Jorgensen et al., 2017; Newton and Manning, 2016). While elimination of the host cell by programmed cell death was once thought to be beneficial in the fight of the host against the infecting pathogen, there is increasing interest in the notion that programmed cell death also plays a role in the innate immune defense against pathogens (Jorgensen et al., 2017). Necroptosis, unlike apoptosis, leads the dying cell to leave behind damage-associated molecular patterns (DAMPs) that further activate the immune system to respond to the pathogen (Jorgensen et al., 2017; Newton and Manning, 2016). Necroptosis is regarded as the optimal form of molecularly-controlled cell death when an inflammatory/immunological response is required as found in pathological situations related to the presence of foreign invaders; a similar view was previously proposed for necrosis, before necroptosis was discovered.

Although one may envision that necroptosis would be instrumental in virtually all cell types, thus far it has been demonstrated as an alternative cell death pathway to apoptosis almost exclusively in immune cells such as macrophages and T-cells, and also microglia, endothelial cells...
and fibroblasts (Newton and Manning, 2016; Weinlich et al., 2016). Moreover, in these cells, especially when caspase-8 activation or recruitment is impaired, necroptosis is known to be engaged downstream of ligation of death and toll-like receptors with ligands such as TNF-α, Fas ligand (FasL), TNF-related apoptosis-inducing ligand (TRAIL), interferon gamma (IFNγ), LPS (lipopolysaccharide produced by Gram-negative bacteria), cluster of differentiation 3/28 (CD3, CD28) and TNF-related weak inducer of apoptosis (TWEAK) (Figure 3.1) (Chan et al., 2003; Degterev et al., 2005; Holler et al., 2000; Matsumura et al., 2000).

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**Figure 3.1. Activation of Necroptosis by Engagement of a Variety of Ligands and Receptors**

Necroptosis activation occurs especially under conditions of impaired caspase-8 recruitment or activation, downstream of ligation of death receptors and toll-like receptors by ligands such as Tumor necrosis factor-α (TNF-α), Fas ligand (FasL), TNF-related weak inducer of apoptosis (TWEAK), TNF-related apoptosis-inducing ligand (TRAIL), LPS (lipopolysaccharide produced by Gram-negative bacteria), cluster of differentiation 3/28 (CD3, CD28), and interferon gamma (IFNγ). Figure reproduced from Vandenabeele et al. (2014) *Path. Hum. Dis.* p.153-161.
However, none of the proposed agonists of necroptosis are responsible for the toxicity of either mouse mSOD1 or sALS astrocyte-conditioned media, discussed below.

### 3.1.1.1. Tumor Necrosis Factor-α (TNF-α)

Given the importance of this ligand to my topic, concentration of TNF-α was measured by enzyme-linked immunosorbent assay (ELISA) in astrocyte-conditioned media (ACM). Strikingly, TNF-α, which engages tumor necrosis factor receptor (TNFR1/2), was detected in the same low levels in wild-type and mSOD1 mouse ACM (Figure 3.2, a) (Nagai et al., 2007). In addition, I performed another ELISA for 31 human cytokines using ACM from primary sALS astrocytes and control astrocytes and did not detect TNF-α (Figure 3.2, d, OD <0.01, below the detection limit of the plate reader/assay). In same assay, I did detect granulocyte macrophage colony-stimulating factor (GM-CSF) (Figure 3.2, d, OD = 0.15-0.2) which is known to be secreted from human astrocytes in primary culture (Choi et al., 2014). Of note, this specific ELISA was meant to compare levels between samples, not to provide absolute values. However the limit of detection of most ELISA reactions are between 0.01 pg/mL to 100 ng/mL (Zhang et al., 2014). Thus, I can conclude that if present, TNF-α might be at least below 100 ng/mL, and GM-CSF is at least above 0.01 pg/mL. Lastly, I noted that spinal motor neurons grown from TNFR1/2 double knockout mice were as susceptible to mSOD1 astrocytes as their wild-type counterparts (Figure 3.2, c). Thus, collectively, I did not have any evidence to implicate either TNF-α or TNFR1/2 in ALS astrocyte-mediated motor neurons death in my studied in vitro models of ALS.
mSOD1 mouse astrocyte toxicity may not involve TNFα, or FASL or TRAIL

**Figure 3.2. Evaluating Ligands and Receptors Associated with Necroptosis Activation**

**a.** ELISA detected the same levels of TNF-α in wild-type (non-transgenic) and SOD1<sup>G93A</sup> ACM

**b-c** Wild-type primary motor neurons (MNs) from E12.5 embryos were co-cultured on astrocyte monolayers from wild-type (non-transgenic NTg) or SOD1<sup>G93A</sup> astrocytes and MN survival was measured after 7 days with or without treatment with 1μg/mL Fas inhibitor Fas-Fc or DR5-neutralizing antibody and no improvement in MN survival was measured. MNs identified by immunostaining for GFP.

**c.** MNs from mice with germline deletion of TNFR1/2 also showed no protection compared to wild-type MNs. MNs identified by SMI32+ immunostaining.

**d.** ELISA against human cytokines on ACM from sALS astrocytes and non-diseased control (NDC) astrocytes showed undetectable levels of TNF-α and no change in IFNγ levels (absorbance 450nm ~0.05, 2-way ANOVA, p= 0.94 ALS vs. NDC). COPD= chronic obstructive pulmonary disorder AD= Alzheimer’s disease. n=3 biological replicates per condition for all experiments (a-e).
3.1.1.2. Fas ligand (FasL)

Although activation of Fas by FasL is a known inducer of motor neuron programmed cell death involving FADD-caspase-8 (Raoul et al., 1999, 2002), in our astrocyte co-culture we have consistently found that Fas inhibition does not rescue motor neuron death from mSOD1 astrocytes (Nagai et al., 2007). Indeed, Fas inhibitor Fas-Fc, which is sufficient to block Fas activation by FasL (Raoul et al., 1999), does not rescue motor neuron death in co-culture with mSOD1 astrocytes (Figure 3.2, b).

3.1.1.3. TNF-related weak inducer of apoptosis (TWEAK)

TWEAK has been shown to be produced by astrocytes and can induce apoptosis in primary motor neurons via a CD163 receptor and caspase-3 dependent mechanism (Bowerman et al., 2015). However, Bowerman et al. (2015) report comparable low levels of TWEAK in the media of wild-type and SOD1G93A astrocyte cultures, and comparable increased levels after stimulation with IFNγ between the two genotypes (Bowerman et al., 2015). Therefore, I postulated that if TWEAK is related to the astrocyte toxicity in our co-culture model via a necroptosis-dependent mechanism, it would likely be as a “priming factor” that would require action of at least a second toxic factor that is differentially produced by SOD1G93A-expressing astrocytes. However, antagonizing this mechanism through genetic deletion of TWEAK or through treatment with antagonistic anti-TWEAK antibody did not improve motor neuron loss in SOD1G93A mice (Bowerman et al., 2015). It is unclear whether TWEAK contributes to a necroptosis-dependent mechanism of motor neuron death. We did not yet look for soluble TWEAK in mSOD1 or sALS astrocytes in our co-culture models.
3.1.1.4. TNF-related apoptosis-inducing ligand (TRAIL)

Two death receptors (DR) respond to TRAIL ligand: DR4, which was not detected in mouse ES-MN by RNA sequencing, and DR5. When the co-culture was treated with DR5-neutralizing antibody, there was no protection from mSOD1 astrocyte induced motor neuron cell death (Figure 3.2, c). ELISA for TRAIL was performed on control and sALS ACM, and there was no significant difference between control and sALS ACM in levels of TRAIL, which was measured by Dr. V. LeVerche, an Associate Research Scientist in the lab. TRAIL was found to be at low levels (10 pg/ml), approaching the detection limits of the assay.

3.1.1.5. Lipopolysaccharide (LPS)

LPS is specifically produced by Gram-negative bacteria and activates TLR4 (Toll-like receptor 4), which leads to necroptosis. As LPS is not produced by astrocytes, it is not a relevant agonist to explore in ACM. However, TLR4 is expressed in mouse ES-MN, determined by RNA sequencing, so engagement of the receptor by other means is possible. While LPS is the most well-studied, in some instances TLR4 has been shown to be activated by other endogenous ligands such as β-defensins, heat shock proteins, fibrinogen cleavage products, and modified forms of low-density lipoprotein (Brubaker et al., 2015, Millien et al., 2013). We did not yet explore TLR4 in the context of motor neurons.

3.1.1.6. Cluster of differentiation (CD)3/CD28

Activation of T-cell receptors (TCR) by CD3 or CD28 engages necroptosis in T-cells. However, TCR (encoded by TRA, TRB, TRG, TRB genes) was not found to be expressed in mouse ES-MN
as determined by RNA sequencing, and therefore I did not consider this mechanism in our study of motor neuron death.

3.1.1.7. Interferon gamma (IFNγ)

IFNγ was undetectable by ELISA in wild-type or mSOD1 ACM (Nagai et al., 2007). In addition, I performed an ELISA on conditioned media from primary sALS astrocytes and non-diseased control (NDC) astrocytes and found no significant difference in levels between NDC and sALS astrocytes (Figure 3.2, e, absorbance at 450 nm ~0.05, 2-way ANOVA, p= 0.94).

Thus, the above data strongly argue that, in the case of our primary motor neuron cultures, necroptosis is the primary driver of motor neuron cell death in response to ALS-associated astrocytes and takes place without stimulation by any known exogenous ligands, ligation of any known receptors, or by first suppressing apoptosis using pan-caspase inhibitors such as zVAD.fmk.

3.1.2. Motor Neuron Cell Death in Response to fALS Astrocytes Is Dependent on Bax Protein

Another unique feature of our model of motor neuron death is the dependence of this process on Bax protein, as shown in Chapter 2. In other cells so far, any generalized mitochondrial-dependent execution mechanism of necroptosis, suggested by some studies (Wang et al., 2012), has not been confirmed by others (Tait et al., 2013). There have been few if any studies evaluating the role of Bax directly in necroptosis. However, downstream execution of necroptosis may be cell-type specific (Vanden Berghe et al., 2015; Morioka et al., 2014; Newton and Manning, 2016; Thornton and Hagberg, 2014)
3.1.3. Role of NF-κB in Motor Neuron Death

A recent study from Przedborski lab (Ikiz et al., 2015), to which I contributed, showed that motor neuron death in response to SOD1G93A-expressing astrocytes was dependent on the canonical pathway of NF-κB. In this work, we showed that not only does the silencing of NF-κB subunit p50 protect motor neurons, but also that NF-κB subunits P50 and P65 bind to DNA in motor neurons upon exposure to media conditioned by ALS astrocytes (Ikiz et al., 2015). Conversely in other necroptosis-competent cells, NF-κB signaling (typically upon TNFR1 activation by TNF-α) is pro-survival; proinflammatory signaling downstream of formation of Complex I requires ubiquitinated RIPK1 and recruitment of NF-kappa-B essential modulator (NEMO), and IκB kinase (IKK) complex components IKK-α and IKK-β (Festjens et al., 2007; Walczak, 2013) (Figure 3.3). Cell death via Complex II formation, which leads to apoptosis or necroptosis depending on the activity of caspase-8, occurs only under conditions where NF-κB signaling is impaired (Walczak, 2013) (Figure 3.3).

One of the first demonstrations of the critical pro-survival role of NF-κB signaling downstream of TNF-α stimulation was in mice with homozygous germline deletion of NF-κB subunit RelA. Deletion of RelA resulted in embryonic lethality and liver degeneration resulting from apoptosis in liver cells (Beg et al., 1995). In addition, embryonic fibroblasts cultured from these mice had decreased viability in response to TNF-α treatment compared to control cells (Beg and Baltimore, 1996).

In contrast, in Ikiz et al. (2015) we have demonstrated that targeting the activation of the canonical pathway of NF-κB that occurs in motor neurons undergoing cell death in response to fALS astrocytes by genetic or pharmacologic inhibition protected motor neurons, which argues
Figure 3.3. Differential Activation of NF-κB Pathway, Apoptosis, and Necroptosis Downstream of Receptor Activation

Upon activation of TNFR1, TRADD and RIPK1 are recruited to the death domain of the receptor to begin formation of Complex I. TRADD recruits TRAF2 and in turn cIAP1/2 is recruited and ubiquitinates various components of Complex I dependent for LUBAC recruitment. LUBAC is responsible for ubiquitination of linear chains on RIPK1 and NEMO, which in turn lead to recruitment and activation of IKK and TAB1/TAK1 complexes required for NF-κB activation and translocation to the nucleus. When RIPK1 is deubiquitinated, Complex II forms and depending on whether caspase-8 is active in the cells, leads to caspase-8/FADD-dependent apoptosis or RIPK3/MLKL-dependent necroptosis.

3.1.4. RIPK1 Did Not Seem to Play a Pro-Survival Role in Motor Neurons

Another feature of our motor neuron necroptosis incongruent with the literature is the fact that for NF-κB having the opposite effect in our cell models of ALS.
RIPK1 does not have a pro-survival role in addition to its role in necroptosis signaling that has been documented by others (Vanden Berghe et al., 2015; Festjens et al., 2007). Indeed, in other models, RNA interference (RNAi) against RIPK1 potentiates death rather than protecting from cell death (Dillon et al., 2014; Kearney et al., 2014). This occurs via loss of pro-survival RIPK1 signaling that is independent of RIPK1 kinase function, but rather dependent on its ubiquitination, which promotes NF-κB pro-survival signaling (Festjens et al., 2007; Newton, 2015) (Figure 3.3). In the case of motor neurons, surprisingly both the kinase inhibition via necrostatin-1 and RNAi silencing of RIPK1 were protective against motor neuron death (Nagai et al., 2007).

Given these differences between our co-culture model of motor neuron death and canonical necroptosis defined in the literature, the specific program invoked by motor neurons in response to SOD1G93A-expressing astrocytes may conserve some elements but also differ in a number of ways. In addition, even in the literature, studies of the downstream mechanisms executing cell death downstream of the necrosome remain elusive. For instance, targeted approaches have revealed the importance of MLKL in necroptosis downstream of RIPK3. Upon activation, MLKL assembles into cation-permeable pores at the plasma membrane and is thought to drive necroptosis by disrupting intracellular ion homeostasis (Cai et al., 2014; Wang et al., 2014; Xia et al., 2016). Whereas MLKL is a critical element downstream of RIPK3 in necroptosis signaling, the RIPK3-dependent phosphoproteome is quite extensive, as one study suggested (Zhong et al., 2014), and downstream signaling elements and are still being uncovered beyond MLKL (Zhong et al., 2014). In addition, it is quite possible that MLKL pores lead to ion disturbances that then act as second-messengers to activate downstream execution elements (Xia et al., 2016). Compared to apoptosis, plasma membrane permeability occurs early in necroptosis, so this certainly could be the case.
Often, we conceptualize that cells execute molecular programs linearly. In reality, a given cellular response reflects the integration of numerous signaling pathways converging. Thus, in light of the previous work from the Przedborski lab in collaboration with the Department of System Biology, I sought to use a bioinformatics meta-analysis of motor neuron RNA-Seq data to gain insights into the molecular network of motor neuron death in response to ALS astrocytes and to determine if motor neuron-specific information about necroptosis could be obtained.

3.2. Results

3.2.1. Reverse Gene Engineering to Decipher the Motor Neuron Death Pathway

In an in vitro model, mouse ES-MN undergo cell death when exposed to ACM from transgenic mice expressing SOD1\textsuperscript{G93A}, a mouse model of ALS (Chapter 2). In Chapter 2, I determined that the mechanism of the motor neuron loss, which occurs between day 3 and day 7 in in vitro culture, conserved core elements of necroptosis. Since mouse ES-MN behave like primary cells (Miles et al., 2004), can be produced in high quantities, and can be purified (Ikiz et al., 2015), they are an optimal system to study the molecular basis of necroptosis.

Ikiz et al. (2015) exposed purified mouse ES-MN to either non-transgenic ACM, ACM from wild-type SOD1 overexpressing astrocytes (a control for overexpression but does not induce motor neuron loss), or ACM from SOD1\textsuperscript{G93A}-expressing astrocytes (Figure 3.4). A time point of 3 days exposure to ACM was selected because it is the point at which SOD1\textsuperscript{G93A}-specific motor neuron loss commences in vitro, and the motor neurons are committed to cell death in response to SOD1\textsuperscript{G93A} ACM, as replacing the media with fresh motor neuron media fails to rescue the motor neuron death (Ikiz et al., 2015). Furthermore, the number of differentially-expressed genes was the
highest at this time point between wild-type and mSOD1 ACM exposure (Ikiz et al., 2015).

Figure 3.4 Graphical Abstract from Ikiz et. al. (2015) Cell. Rep.

Purified healthy mouse ES-MN were exposed to ACM from transgenic (Tg) SOD1<sup>G93A</sup>, wild-type SOD1 overexpressing, or wild-type (non-Tg) astrocytes. After 3 days exposure, RNA was extracted and quantified using RNA sequencing. By interrogating the mouse brain interactome with the gene expression signatures built from RNA sequencing, candidate master regulators were identified. Candidate genes were then assessed in vitro on the ability of their silencing to protect against SOD1<sup>G93A</sup> specific astrocyte toxicity to motor neurons.

The approach taken by Ikiz et al. (2015) did not use gene expression profiling to look at altered expression in selected target genes among conditions. Instead, using RNA-Seq data
obtained at 3 days in vitro, relative levels of all expressed transcripts for each condition were quantified to generate a gene expression signature (3d-GES). Then, the entire set of transcriptional changes was used to infer changes in transcriptional regulators, specifically "master regulators," or the transcription factors that are necessary and sufficient to explain the studied phenotype. This was achieved using a large dataset of known cell-specific interactions: the mouse brain interactome (reverse-engineered from mouse whole-brain gene expression profiles) and the Master Regulator Interference algorithm (MARINa) (Lefebvre et al., 2010). MARINa interfaces between the transcriptional data and the mouse brain interactome to infer the set of master regulators that is statistically most likely to explain the 3d-GES. This systems approach has been validated in a number of cell types and contexts (Carro et al., 2010; Ikiz et al., 2015; Lefebvre et al., 2010).

While Ikiz et al. (2015) generated and validated a number of candidate master regulators (MRs) of motor neuron cell death utilizing this approach, their analysis addressed a different set of questions than those in my current study. Here, in light of our findings using targeted approaches involving necroptosis machinery, I sought to determine whether there was evidence of necroptosis in the signature generated by Ikiz et al. (2015).

3.2.2. The Signature Was Enriched for an Available Necroptosis Signature

Hitomi et al. (2008) induced necroptosis in a mouse fibrosarcoma cell line (L929), and used an siRNA-based repressor screen to identify positive regulators of necroptosis in those cells, e.g., those genes that when downregulated in expression were sufficient to protect cells from zVAD-induced necroptosis. Using the list of necroptosis drivers generated by Hitomi et al., and specifically those 40 genes that have neuronal expression, I discovered that the motor neuron death signature generated (3d-GES) by Ikiz et al. (2015) was significantly enriched for neuronal drivers
of necroptosis (Figure 3.5, normalized enrichment score [NES]= 6.89, p= 2.7x10^{-12}). Importantly, Hitomi et al. did not perform their experiments in neurons, but rather derived the 432 genes they identified as zVAD-induced necroptosis drivers from L929 cells, and identified those that also are expressed in neurons. Despite the limitations of the list in that it may not indeed reflect the signaling specific to neurons undergoing necroptosis because it was derived from a cancer cell line, my enrichment was promising. Table 3.1 details the list of genes driving the positive enrichment.

Figure 3.5. The 3d-GES is Enriched for a Neuronal Necroptosis Gene Set

Gene Set Enrichment Analysis for 40 neuron-specific necroptosis genes from Hitomi et al. (2008) on the 3d-GES. The maximum value of the curve determines the enrichment score. The vertical lines indicate the projection of the neuron-specific necroptosis genes on the sorted GES. The normalized enrichment score (NES) and associated p-value is shown.
### Table 3.1 Gene Set Enrichment Analysis for 3d GES Against Known Necroptosis Drivers

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<td>Tceb1</td>
<td>0.82</td>
<td>0.413</td>
<td>0.459</td>
<td>NO</td>
</tr>
<tr>
<td>107831</td>
<td>Bix1</td>
<td>0.57</td>
<td>0.567</td>
<td>0.596</td>
<td>NO</td>
</tr>
<tr>
<td>102693</td>
<td>Phidc1</td>
<td>-2.46</td>
<td>0.0139</td>
<td>0.0293</td>
<td>NO</td>
</tr>
<tr>
<td>20420</td>
<td>Shd</td>
<td>-1.68</td>
<td>0.0935</td>
<td>0.139</td>
<td>NO</td>
</tr>
<tr>
<td>67120</td>
<td>Ttc14</td>
<td>-1.38</td>
<td>0.168</td>
<td>0.24</td>
<td>NO</td>
</tr>
<tr>
<td>97387</td>
<td>Strn4</td>
<td>-1.32</td>
<td>0.187</td>
<td>0.258</td>
<td>NO</td>
</tr>
<tr>
<td>17136</td>
<td>Mag</td>
<td>-0.9</td>
<td>0.368</td>
<td>0.446</td>
<td>NO</td>
</tr>
<tr>
<td>20370</td>
<td>Sez6</td>
<td>-0.88</td>
<td>0.381</td>
<td>0.448</td>
<td>NO</td>
</tr>
<tr>
<td>67952</td>
<td>Tomm20</td>
<td>-0.7</td>
<td>0.482</td>
<td>0.521</td>
<td>NO</td>
</tr>
<tr>
<td>14588</td>
<td>Gfra4</td>
<td>-0.32</td>
<td>0.747</td>
<td>0.766</td>
<td>NO</td>
</tr>
<tr>
<td>50524</td>
<td>Sall2</td>
<td>-0.1</td>
<td>0.918</td>
<td>0.918</td>
<td>NO</td>
</tr>
</tbody>
</table>

**Legend:** 40 neuron-specific necroptosis genes from Hitomi et al. (2008) indicating their differential expression at 3-days after mSOD1 ACM treatment (Z-score, p-value and FDR). The Ledge column indicates the fraction of the necroptosis genes that are in the leading-edge when their enrichment on the 3d-GES was computed by gene set enrichment analysis. Table reproduced from Ikiz et al. (2015).

The genes driving the positive enrichment in the gene set in this analysis were candidates
for drivers of necroptosis in our model. However, I also wanted to consider in our 3d-GES the entire list of 432 genes Hitomi et al. identified as zVAD-induced necroptosis drivers in mouse L929 cells. I wanted to expand beyond the list of 40 “neuronal” drivers to also consider the 432-gene list because I wanted to remove the database-derived bias of assuming the genes were expressed in neurons. Databases considering expression levels usually rely on basal conditions rather than what could be happening in pathological situation. In addition, the Hitomi et al. study was performed in 2008 and improved annotations could have been made since then.

To expand the gene set for analysis, I filtered the list of genes in the 3d-GES that were increased in expression in the mSOD1 ACM condition (positive two-tail Student’s t-test statistic in this case), because the Hitomi et al. screen was biased for positive regulators of necroptosis and my assumption was that these would increase in expression in our signature. I used cutoff values of p<0.005 and false discovery rate (FDR)<0.05, which from consulting with colleagues are commonly-accepted cutoffs for this type of analysis. This generated 689 genes upregulated genes in the 3d-GES mSOD1 ACM condition. I then determined which genes coincided with the list of 432 genes Hitomi et al. identified as necroptosis drivers. Indeed, of the 26 genes that overlapped the Hitomi et al. screen and Ikiz et al. (2015) mSOD1-ACM responsive (increased) gene expression signature, 11 were not originally deemed neuronally expressed by Hitomi et al. (Table 3.2, indicated by “N”). An annotated table of these 26 genes is in Table 3.2.
Co-occurring necroptosis hits from Hitomi et al. with increased expression in mSOD1 ACM-induced motor neuron death signature

<table>
<thead>
<tr>
<th>GeneID</th>
<th>Symbol</th>
<th>Annotation</th>
<th>t-statistic</th>
<th>3d-GES p-value</th>
<th>FDR</th>
<th>432 list vZAD hits</th>
<th>40 neuronal list necroptosis drivers</th>
</tr>
</thead>
<tbody>
<tr>
<td>72976</td>
<td>Cnih3</td>
<td>comichon homolog 3 (Drosophila)</td>
<td>5.995</td>
<td>1.98E-06</td>
<td>0.000436</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>14702</td>
<td>Gng2</td>
<td>guanine nucleotide binding protein (G protein), gamma 2 subunit</td>
<td>5.524</td>
<td>7.02E-06</td>
<td>0.000921</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>23723</td>
<td>Gira2</td>
<td>glycine receptor, alpha 2 subunit</td>
<td>5.497</td>
<td>7.58E-06</td>
<td>0.000962</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>269190</td>
<td>Dpp10a</td>
<td>disintegrin and metalloproteinase 10</td>
<td>5.296</td>
<td>1.52E-05</td>
<td>0.00154</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>20907</td>
<td>Stx1a</td>
<td>syntaxin 1A (brain)</td>
<td>4.917</td>
<td>3.63E-05</td>
<td>0.00277</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>14784</td>
<td>Grb2</td>
<td>growth factor receptor bound protein 2</td>
<td>4.745</td>
<td>5.79E-05</td>
<td>0.00385</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>23984</td>
<td>Pde10a</td>
<td>phosphodiesterase 10A</td>
<td>4.736</td>
<td>5.98E-05</td>
<td>0.00391</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>218793</td>
<td>Ubc4e2</td>
<td>ubiquitin-conjugating enzyme E2E 2 (UBC4/S homolog, yeast)</td>
<td>4.665</td>
<td>7.19E-05</td>
<td>0.00437</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>103967</td>
<td>Dnm3</td>
<td>dynamin 3</td>
<td>4.39</td>
<td>0.000151</td>
<td>0.00693</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>26434</td>
<td>Prnd</td>
<td>prion protein dublet</td>
<td>4.255</td>
<td>0.000217</td>
<td>0.00865</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>14406</td>
<td>Gabrg2</td>
<td>gamma-aminobutyric acid (GABA-A) receptor, subunit gamma 2</td>
<td>4.03</td>
<td>0.000397</td>
<td>0.0125</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>23908</td>
<td>Hs2st1</td>
<td>heparan sulfate 2-O-sulfotransferase 1</td>
<td>3.982</td>
<td>0.000452</td>
<td>0.0134</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>244238</td>
<td>Mrpre</td>
<td>MAS-related GPR, member E</td>
<td>3.95</td>
<td>0.000492</td>
<td>0.014</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>12941</td>
<td>Pcdha5</td>
<td>protocadherin alpha 5</td>
<td>3.917</td>
<td>0.000537</td>
<td>0.0148</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>66237</td>
<td>Atp6v1g2</td>
<td>ATPase, H+ transporting, lysosomal V1 subunit G2</td>
<td>3.887</td>
<td>0.000582</td>
<td>0.0155</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>98403</td>
<td>Zfp451</td>
<td>zinc finger protein 451</td>
<td>3.78</td>
<td>0.000814</td>
<td>0.0192</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>14172</td>
<td>Fgf18</td>
<td>fibroblast growth factor 18</td>
<td>3.712</td>
<td>0.000922</td>
<td>0.0206</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>331474</td>
<td>Rgs4</td>
<td>retrotansposon gag domain containing 4</td>
<td>3.612</td>
<td>0.00112</td>
<td>0.0249</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>68236</td>
<td>Mtum</td>
<td>matrin</td>
<td>3.567</td>
<td>0.00135</td>
<td>0.0269</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>16476</td>
<td>Jun</td>
<td>Jun oncogene</td>
<td>3.508</td>
<td>0.00167</td>
<td>0.0296</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>20261</td>
<td>Sema7a</td>
<td>semaphorin domain (lg), and GPI membrane anchor, (semaphorin) TA</td>
<td>3.324</td>
<td>0.00252</td>
<td>0.0407</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>269180</td>
<td>Inpp4a</td>
<td>inositol polyphosphate-4-phosphatase, type I</td>
<td>3.289</td>
<td>0.00275</td>
<td>0.0431</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>26913</td>
<td>Gprin1</td>
<td>G protein-regulated inducer of neurite outgrowth 1</td>
<td>3.276</td>
<td>0.00284</td>
<td>0.044</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>209497</td>
<td>Tmem164</td>
<td>transmembrane protein 164</td>
<td>3.244</td>
<td>0.00308</td>
<td>0.0461</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>223618</td>
<td>Nmnat2</td>
<td>nicotinamide nucleotide adenyltransferase 2</td>
<td>3.223</td>
<td>0.00325</td>
<td>0.0473</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>54861</td>
<td>Nap113</td>
<td>nucleosome assembly protein 1-like 3</td>
<td>3.152</td>
<td>0.00388</td>
<td>0.053</td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>

Legend: Genes identified by Ikiz et al. (2015) with increased mRNA expression following 3 days exposure to SOD1(G93A) astrocyte conditioned media, based on statistical criteria: false discovery rate (FDR) (<0.05) and p-value (<0.005), that also were identified by Hitomi et al. (2008) as necroptosis drivers in L929 cells. "432 list vZAD hits" = the full list of genes identified by Hitomi et al. (2008) as necroptosis drivers. "40 neuronal list" = the subset of genes identified by Hitomi et al. (2008) that were deemed as "neuronally-expressed." "Y" = yes, "N" = no.
Of the list, only *zfp451* was a master regulator of motor neuron cell death identified by Ikiz et al. (2015) with FDR < 0.05, and it was ranked #117 (NES = 2.86, p = 0.00423, FDR = 0.0486) with a predicted increase in activity (positive NES). Also, only *zfp451* and *jun* have known roles as transcription factors, and *nap1l3* is a nucleosome assembly factor that could be involved in transcriptional regulation.

*Zfp451* (zinc finger protein 451), stood out as a transcription factor that was increased in the motor neuron death signature, identified as a necroptosis driver in mouse fibrosarcoma cells (Hitomi et al., 2008), and was identified as a master regulator of motor neuron cell death (Ikiz et al., 2015). In addition, the regulon of *zfp451* contains *inpp4a* (inositol polyphosphate-4-phosphatase type 1), which was indeed increased in our signature and was also identified as a necroptosis driver by Hitomi et al. (Table 3.2). While not much is known about *zfp451*, its regulon, which contains 204 genes, was enriched in functional pathways that could be related to neurodegeneration and to necroptosis. *Inpp4a* was interesting in its own right because mutations in or silencing of *inpp4a* lead to neurodegeneration in mice, establishing a role of *inpp4a* in neuroprotection (Nystuen et al., 2001; Sasaki et al., 2010). Inpp4a protein is a phosphatase regulating phosphatidylinositol-3,4-bisphosphate (PTdIns(3,4)P₂) that may mediate neuroprotection through suppression of NMDAR (N-methyl-D-aspartate-type glutamate receptor) mediated excitotoxicity by negatively regulating NMDAR density at the synapse (Sasaki et al., 2010). Furthermore, when I performed analysis using String software, among the top five Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched in the *zfp451* regulon were Alzheimer’s disease, Huntington’s disease, and Parkinson’s disease (Table 3.3). As these are also neurodegenerative disorders, experimental validation is warranted to determine what relevance, if any, an increase in activity *zfp451* could play in ALS and these other diseases. In addition, non-
Alcoholic fatty liver disease (NAFLD) was also in the top five KEGG pathways enriched in the regulon of zfp451 (Table 3.3). Interestingly, necroptosis has also been linked in mechanisms of NAFLD (Afonso et al., 2015). One clue into the mechanisms represented by the regulon of zfp451 was the enrichment for biological process, including mechanisms involving the mitochondria and responses to cellular stress in the top five KEGG pathways (Table 3.4). Taken together, experimental validation of zfp451 in in vitro and in vivo models of ALS and necroptosis could provide more clues as to the link between this transcription factor and motor neuron death in ALS.

---

Table 3.3. KEGG Pathways Enriched in the zfp451 Regulon

<table>
<thead>
<tr>
<th>#ID</th>
<th>Pathway description</th>
<th>gene count</th>
<th>FDR</th>
<th>matching proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>5010</td>
<td>Alzheimer's disease</td>
<td>9</td>
<td>0</td>
<td>Adam17,Cacna1d,Gnaq,Ndufa6,Ndufa7,Ndufc1,Ndufs5,Ndufv2,Uqcr11</td>
</tr>
<tr>
<td>4932</td>
<td>Non-alcoholic fatty liver disease (NAFLD)</td>
<td>8</td>
<td>0.01</td>
<td>Bcl2l11,Mapk8,Ndufa6,Ndufa7,Ndufc1,Ndufs5,Ndufv2,Uqcr11</td>
</tr>
<tr>
<td>190</td>
<td>Oxidative phosphorylation</td>
<td>7</td>
<td>0.01</td>
<td>Atp6ap1,Ndufa6,Ndufa7,Ndufc1,Ndufs5,Ndufv2,Uqcr11</td>
</tr>
<tr>
<td>5016</td>
<td>Huntington's disease</td>
<td>8</td>
<td>0.01</td>
<td>Gnaq,Ndufa6,Ndufa7,Ndufc1,Ndufs5,Ndufv2,Polr2i,Uqcr11</td>
</tr>
<tr>
<td>5012</td>
<td>Parkinson's disease</td>
<td>7</td>
<td>0.01</td>
<td>Ndufa6,Ndufa7,Ndufc1,Ndufs5,Ndufv2,Park7,Uqcr11</td>
</tr>
<tr>
<td>4723</td>
<td>Retrograde endocannabinoid signaling</td>
<td>6</td>
<td>0.01</td>
<td>Cacna1d,Gabra3,Gabbr2,Gnaq,Gria4,Mapk8</td>
</tr>
<tr>
<td>460</td>
<td>Cyanoamino acid metabolism</td>
<td>2</td>
<td>0.05</td>
<td>Ggt5,Shmt1</td>
</tr>
</tbody>
</table>

---

Table 3.4. Biological Processes Represented in zfp451 Regulon

<table>
<thead>
<tr>
<th>#ID</th>
<th>pathway description</th>
<th>gene count</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO.0007005</td>
<td>mitochondrion organization</td>
<td>13</td>
<td>0.0101</td>
</tr>
<tr>
<td>GO.0009987</td>
<td>cellular process</td>
<td>121</td>
<td>0.0101</td>
</tr>
<tr>
<td>GO.0010273</td>
<td>detoxification of copper ion</td>
<td>3</td>
<td>0.0101</td>
</tr>
<tr>
<td>GO.1990169</td>
<td>stress response to copper ion</td>
<td>3</td>
<td>0.0101</td>
</tr>
<tr>
<td>GO.0044765</td>
<td>single-organism transport</td>
<td>40</td>
<td>0.0131</td>
</tr>
<tr>
<td>GO.0033554</td>
<td>cellular response to stress</td>
<td>27</td>
<td>0.0205</td>
</tr>
<tr>
<td>GO.0006996</td>
<td>organelle organization</td>
<td>40</td>
<td>0.0222</td>
</tr>
<tr>
<td>GO.0008152</td>
<td>metabolic process</td>
<td>94</td>
<td>0.0222</td>
</tr>
<tr>
<td>GO.0044237</td>
<td>cellular metabolic process</td>
<td>83</td>
<td>0.0235</td>
</tr>
<tr>
<td>GO.0006839</td>
<td>mitochondrial transport</td>
<td>7</td>
<td>0.0309</td>
</tr>
<tr>
<td>GO.0016043</td>
<td>cellular component organization</td>
<td>54</td>
<td>0.0488</td>
</tr>
</tbody>
</table>
Of those directly related to cell death, one gene of particular interest was \( \text{jun} \), which in some contexts is anti-apoptotic. While \( \text{jun} \)—which encodes c-Jun, a subunit of transcription factor AP-1—has roles in cell cycle progression in G1, it is also involved in anti-apoptotic signaling through a distinct mechanism (Wisdom et al., 1999). Interestingly, in a study from Kostic et al., (1997), the authors used c-Jun as a cell stress marker in transgenic SOD1\(^{G93A}\) mice and found that, at symptomatic stage, many spinal motor neurons exhibited a strong nuclear c-Jun immunoreactivity. Several years later, as a post-doctoral researcher in our lab, Diane Re infected cultured motor neurons with an adenovirus expressing a shRNA against c-Jun and found that silencing this gene target dramatically enhanced motor neuron susceptibility to astrocyte ACM; however, this study was never pursued in greater detail. In other settings, it has been shown that c-jun-deficient fibroblasts showed enhanced sensitivity to UV-induced apoptosis, and TNF-\( \alpha \)-induced apoptosis (when pro-survival NF-\( \kappa \)B was also inactivated) suggesting a protective role (Wisdom et al., 1999). Also, c-Jun is increased in neuronal response to injury and is involved in promoting survival of axotomized neuronal cell bodies and axonal regeneration in facial motor neurons following injury (Ruff et al., 2012).

The other genes, while not driving transcription, were predicted to be response elements. Cellular component analysis of the 26-gene list using String-db software identified an enrichment for synapse-associated proteins (\( \text{cnih3}, \text{glra2}, \text{stx1a}, \text{dnm3}, \text{gabrg2}, \text{atp6v1g2}, \text{nmnat2}, \text{FDR} = 0.0063 \)) and membrane-associated proteins (\( \text{cnih3}, \text{gng2}, \text{glra2}, \text{dpp10}, \text{grb2}, \text{gabrg2}, \text{atp6v1g2}, \text{FDR} = 0.0454 \)).

Whereas generating a list using transcriptionally-responsive genes as I have done could lead to interesting insights when compared to a known list of necroptosis drivers from the
literature, in general using transcriptionally-responsive genes is not typically very useful to identify true drivers of a given cellular phenotype (e.g., “master regulators”). Using the gene expression signature and powerful systems based techniques, the activity of key transcriptional drivers of cell fate changes could be inferred due to large scale changes in those responsive genes that they regulate (e.g., their regulon). In many cases, these key drivers themselves might not be the most differentially expressed genes in the signature and might be regulated through post-translational modifications, so their involvement could be missed in traditional screens.

I thus quickly turned my attention to the master regulator analysis in order to gain insights into the drivers of the motor neuron death phenotype in our model. This analysis allowed me to better predict global changes mediated by master regulators, rather than comparing signatures at the individual transcript level, which could result in a bias toward nonspecific responsive elements owing to secondary effects and biological noise.

3.2.3 Re-Analysis of the moto neuron signature 3d-GES

In the time since Ikiz et al. (2015) published their report, there have been several improvements in the methods used to infer key transcriptional regulators based on RNA-sequencing data and a cell context-specific interactome. The updated virtual inference of protein activity by enriched regulon analysis (msVIPER) method (compared to MARINA) interrogates the mouse brain interactome using the RNA-sequencing data (Alvarez et al., 2016). Importantly, improvements have been made in the strength of the protein activity inference and the generation of a null model to infer candidates. The null model is an assumption of the null hypothesis that is computed using random shuffling of the sample labels. This takes into account variability (e.g., experimental noise) that
occurs from running the computational analysis itself by making multiple permutations of the data. By running an enrichment against the null model, if the variance between control and experimental is above the threshold set by the null model, e.g., the noise created from the permutations of randomly-shuffled data, the significance can be inferred with reduced likelihood of a false-positive and false-negative. Therefore, I decided rather than exploring master regulators generated by Ikiz et al. (2015), to re-analyze the RNA-seq data using the most updated and robust methods available. Additionally, it should be noted that Ikiz et al. (2015) ran an updated version of MARINa (a beta version of msVIPER) and thus generated a null model by permuting the samples uniformly at random 1,000 times in order to infer statistical significance. However, the robustness of this analysis was never explored. Therefore, by re-analyzing the data, I could also answer the question as to the reproducibility of the analysis by independent generation of the null model.

In collaboration with Alessandro Vasciaveo, Mariano Alvarez and Andrea Califano, the 2015 RNA-sequencing data were re-analyzed using the updated methods. All 23 of Ikiz et al. (2015) MRs occurred in the top 50 MRs of the re-analysis with the same predicted directionality of the change, but with slightly different rankings (Figure 3.6, highlighted in yellow). However, despite the coherence with the previous analysis, a number of novel predicted MRs of astrocyte-mediated motor neuron cell death were identified in the new analysis that warranted consideration, discussed below.
Figure 3.6. Inferred Drivers of mSOD1 Astrocyte-Induced Motor Neuron Death

Top 50 statistically significant master regulators (MRs) (p<0.001, FDR<0.05) inferred by msVIPER analysis of the mouse brain interactome, using the 3-day signature of MNs exposed to mSOD1 ACM. X-axis represents all genes, sorted from the largest decrease to the largest increase in expression. For each MR, activated and repressed ARACNe-inferred targets are shown as red and blue bars, respectively, to indicate that msVIPER considered them separately to compute MR significance. MRs are sorted from those with greatest predicted increase in activity (top) to those with greatest predicted increase in activity (bottom). Top 10 MRs with increase in activity were zfp697, sox5, epas1, zdhhc2, app, zfp235, npas2, ze3h13, prdm15, camta1. Top 10 MRs with decrease in activity were tgif2, hoxc6, zcchc3, nfkb1, zfp646, irx5, icf3, hmgdb2, tgif1, and gfi1. Highlighted (yellow) values indicate the previously inferred top 23 MRs most significant by Ikiz et al. (2015). Act= activity inferred by msVIPER by absolute normalized enrichment score (heatmap: red=increase blue=decrease). Exp= expression of TF (z-score) expressed as heat map (red= increase, blue=decrease, white=no change).
3.2.3.1. Master Regulators with Predicted Increases in Activity

Of the top 10 MRs that had predicted increases in activity from the new analysis, zfp697, sox5, npas2, zc3h13, and prdm15, were not ranked as such in Ikiz et al. (2015); therefore, their contributions to driving the motor neuron cell death signature were not previously discussed or validated. Of the top 10 MRs from the new analysis, Ikiz et al. (2015) validated epas1 and zdhhc2 as astrocyte-specific motor neuron death drivers, and app, zfp235, and camta1 were not confirmed as astrocyte-specific motor neuron death drivers.

3.2.3.1.a. Novel master regulators with predicted increases in activity in the MN death signature

zfp697: Not much is known about zinc finger protease-697 (zfp697), but according to the Allen Brain Atlas, this gene is expressed in neurons in the spinal cord of adult mice.

sox5: SRY-related HMG-box-5 (Sox5) has known roles in nervous system development and affects the proliferative capacity of neuronal progenitors. It is involved in cell fate and promotes terminal differentiation, e.g., cell cycle exit, of neurons in the chick spinal cord, and was found to be expressed by islet 1/2+ dorsal interneurons and a small subpopulation of the islet 1/2+ motor neurons (Martinez-Morales et al., 2010).

npas2: Neuronal PAS Domain Protein 2 (Npas2) positively regulates cell survival in cancer cells by leading to a cascade of events that inhibit apoptosis through transcriptional upregulation of CDC25A, a phosphatase that dephosphorylates CDK2/4/6 and Bcl-2 (Yuan et al., 2017). Npas2 has been shown to positively regulate cell cycle and DNA repair genes in the context of cancer (Hoffman et al., 2008). According to the Allen Brain Atlas, there is a neuronal pattern of expression in adult mouse brain, and it is present in the spinal cord at low levels in neurons. In neurons it has been shown to be a circadian clock responsive element (Reick, 2001).
**zc3h13**: Zinc Finger CCCH-Type Containing 13 (Zc3h13) positively regulates NF-κB activation, as knockdown of zc3h13 inhibited NF-κB activation following LMP1, TNF-α and IL-1β stimulation in HEK293 cells. In addition, MLKL was important for NF-κB activation downstream of LMP1 and IL-1β activation (Gewurz et al., 2012). According to genecards database zc3h13 is expressed in the spinal cord.

**prdm15**: PR/SET Domain 15 (Prdm15) has an unknown function but is expressed in the mouse spinal cord in neurons according to the Allen Brain Atlas.

3.2.3.2. Master Regulators with Predicted Decreases in Activity

Of the top 10 MRs from the new analysis that had predicted decreases in activity, tgif2, hoxc6, zcchc3 and gfi1 were not ranked as such in Ikiz et al. (2015), and therefore their contribution to driving the motor neuron cell death signature was not discussed or validated. However, of the same top 10 MRs, Ikiz et al. (2015) validated nfkb1, tcf3, and tgif1 as astrocyte-specific motor neuron death drivers, and zfp646, irx5, and hmgb2 were not confirmed as astrocyte-specific motor neuron death drivers.

3.2.3.2.a. Novel master regulators with predicted decreases in activity in the MN death signature

**tgif2**: Loss of both TGF-β Induced Factor Homeobox 1 (Tgif1), previously validated by Ikiz et al. (2015) as a master regulator of astrocyte-specific motor neuron death, and Tgif2 in mice is lethal on some backgrounds (Melhuish et al., 2016). Tgif1/Tgif2 are transcriptional repressors of TGF-β signaling, so if the activity of Tgif1/Tgif2 was predicted to decrease, which could mean that TGF-β signaling is active in motor neurons undergoing cell death.
**hoxc6**: Homeobox C6 (*Hoxc6*) is a determinant of motor neuron pool diversity within the LMC of the spinal cord during development (Lacombe et al., 2013).

**zcchc3**: Not much is known about Zinc Finger CCHC-Type Containing 3 (*Zcchc3*), but it is neuronally expressed in mouse spinal cord according to the Allen Brain Atlas.

**gfi1**: Gfi-1 inhibits NLRP3 inflammasome activation and proinflammatory signaling in macrophages. NLRP3 inflammasome activation is an intrinsic signal upstream of RIPK3 that mediates the proinflammatory role of RIPK3 rather than cell death through extrinsic activation of necroptosis through a death receptor (Lawlor et al., 2015). Downregulation of *gfi1* was shown to promote inflammation-linked metastasis of colorectal cancer (Xing et al., 2017). Gfi-1 was also shown to inhibit apoptosis and inflammation in a Bax-dependent manner (Grimes et al., 1996). During TGF-β signaling, Gfi-1 is downregulated (Chalmin et al., 2012). The transcript is not highly expressed in mouse spinal cord neurons according to the Allen Brain Atlas.

### 3.2.3.3. Emerging Themes in the Astrocyte-Mediated Motor Neuron Cell Death Signature

#### 3.2.3.3.a. NF-κB activity

Besides *NFkB1* itself (one of the NF-κB subunits) predicted as being a driver of the motor neuron death signature, validated by Ikiz et al., *zc3h13* may also positively regulate the NF-κB signaling pathway (Gewurz et al., 2012). There have been links to NF-κB signaling in neurodegeneration, but namely in a protective role of activation rather than a deleterious role (Mattson and Meffert, 2006). It remains unclear why experimental validation showed that reducing levels of NF-κB or inhibiting its activity was neuroprotective in this case. However, some evidence has accumulated
that under conditions of cellular stress, NF-κB could promote cell death. One mechanism proposed is that under stress conditions, the RelA subunit of NF-κB translocates to the nucleolus and promotes apoptosis through promoting nucleophosmin (Npm) translocation to the cytoplasm, which in turn mediates mitochondrial accumulation of Bax (Khandelwal et al., 2011). Given that Bax was instrumental in our model, this was an interesting avenue to explore.

3.2.3.3.b. TGF-β signaling

Three of the top predicted drivers of motor neuron cell death were involved in TGF-β signaling according to the literature, namely Gfi1, and Tgff1/Tgif2. The predicted decreased activity of these three elements was consistent with an increase in TGF-β signaling based on their roles, as I discussed, in that tgif1/tgif2 both repress TGF-β signaling, and Gfi-1 is downregulated in response to TGF-β signaling. Furthermore smad3, which was in the top 11 MRs with increased activity in mSOD1 condition, was indeed activated by TGF-β signaling (Derynck and Zhang, 2003).

Despite much of this evidence in the signature pointing toward TGF-β activation, I did not, however, detect TGF-β in sALS astrocyte-conditioned medium by ELISA (absorbance 450 < 0.01). So if there was evidence of increased TGF-β signaling in response to astrocytes in our model, it might be due to ligand-independent increase in activity. TGF-β signaling downstream of receptor activation is highly regulated by SMAD proteins binding to microtubules; one study showed activated TGF-β transcriptional pathways independent of treatment with exogenous TGF-β when microtubules were destabilized (Dong et al., 2000).

TGF-β signaling has a number of cellular functions, including promoting neurogenesis during development. There has been some evidence that reduced TGF-β signaling in neurons contributes to age-dependent neurodegeneration in a model of Alzheimer’s disease (Tesseur et al.,
There is also a degree of crosstalk between TGF-β signaling and NF-κB, mediated by TAK1 kinase (TGF-β activated kinase 1) and SMAD7 (Bitzer et al., 2000; Freudlsperger et al., 2013).

3.2.3.3.c. Motor neuron development and maturation

A number of predicted drivers of motor neuron cell death were involved in motor neuron development and diversity, including Hoxc6, Sox5. In addition, Hoxa9 was not in the top 10 but was in the top 50 predicted MRs of motor neuron death, and it also functions in motor neuron differentiation during development. In addition, although the link to motor neurons is less clear, tgif1 and tgif2 are also known homeobox proteins with roles in development. It is unclear what role, if any these factors may play post-development. One proposed mechanism of neuronal death is that paradoxical re-activation of cell cycle machinery in neurons may act as a cell death signal (Greene et al., 2004, 2007).

There is a notion that under conditions of normal stress, mitotic cells have a tendency to re-enter the cell cycle. However, in neurons, which are post-mitotic, this attempt at re-entry is a failed event and one possible mechanism of neurodegeneration. This is just one example whereby a tightly-controlled developmental physiological mechanism could by a pathological process become re-activated and detrimental.

Pathological reactivation could also tie in the involvement of Npas2, which has known roles in cell cycle regulation, although it is not a homeobox protein. Indeed, as NPAS2 has been shown to regulate the cell cycle in mitotic cells via a CDC25A-dependent mechanism (Yuan et al., 2017), CDC25A was indicated in neurons as a response element to genotoxic stress, and expression of CDC25A was shown to promote cortical neuron cell death even in the absence of DNA damage (Zhang et al., 2006).
Another similar yet independent possibility is that neurons under stress conditions start to reduce levels of mature markers of differentiation in order to conserve energy from transcription and translation. Mature neurons may be more resistant to cell death than immature neurons (Kole et al., 2013). This transition in turn could re-activate elements involved in cell fate and maturation and explain that what we were seeing was a consequence of the cells under stress that eventually succumb to cell death.

3.3. Discussion

3.3.1. Summary of Key Findings

3.3.1.1. Necroptosis Activation in ALS Differs from Other Models of Necroptosis

While motor neuron death in response to ALS-associated astrocytes may involve necroptosis core machinery such as RIPK1, RIPK3, and MLKL, upstream activators or downstream executioners of cell death may be specific to motor neurons compared to other cellular models. In looking for known ligands and receptors at the plasma membrane that have been known to engage necroptosis downstream machinery in some conditions, none of the conserved elements including TNF-α, Fas, TRAIL, or IFNγ seemed to be involved in our co-culture model. We have yet to explore TWEAK in our model, but others have shown that there is some level of TWEAK expressed by both wild-type and mSOD1 astrocytes. However, it did not seem to be necessary for the death of motor neurons in vivo as pharmacologic or genetic inhibition of TWEAK did not rescue motor neuron loss in SOD1<sup>G93A</sup> animals (Bowerman et al., 2015). Other key differences with canonical necroptosis activation was that Bax was instrumental in our model of motor neuron death, NF-κB
signaling led to cell death rather than survival or inflammation, and RIPK1 was not necessary for motor neuron cell survival in addition to having a role in cell death.

3.3.1.2. Reverse Gene Engineering to Decipher Motor Neuron Death Pathway

At the transcriptional level, the motor neuron death signature generated (3d-GES) by Ikiz et al. was significantly enriched for neuronal drivers of necroptosis. I also generated a list of genes that are both upregulated in the motor neurons after exposure to mSOD1 ACM and were identified as drivers of necroptosis in L929 cells by Hitomi et al. (2008). Using updated techniques, I re-analyzed the 3d-GES by Ikiz et al. (2015) by reverse gene engineering and identified new candidate master regulators that could be driving motor neuron cell death. Three themes emerged from the list of motor neuron death drivers: NF-κB was activated, TGF-β signaling seemed to be activated, and genes involved with motor neuron development and activation were activated. The role of these mechanisms, whether they were activated as a neuroprotective response, or indeed driving death, will need to be explored in future studies.

Determining the activation status of TAK1 during motor neuron death could be another interesting lead to follow. TAK1 activation can follow both TGF-β and TNF-α activation of surface receptors (Freudlsperger et al., 2013). Following TNF-α activation, TAK1 could lead to NF-κB activation through phosphorylation of IKK in a RIPK1-dependent manner (Ea et al., 2006). TAK1 activation can also play a role in RIPK3-mediated necroptosis after TNF receptor stimulation depending on the cellular context (Morioka et al., 2014). Given that we saw evidence of NF-κB and TGF-β signaling and necroptosis activation in our model, and TAK1 was involved in the activation of all three of these pathways, evaluating the status of and/or requirement for activation of TAK1 kinase during ALS astrocyte-mediated motor neuron death could provide
another clue into the mechanism.

3.3.2. Validation of Candidates and Exploration of Themes

The candidates I have identified were inferential and require biological validation in future studies to determine relevance. Candidates will first be validated for their effects on motor neuron death markers and survival using pharmacologic approaches (when available), and genetic approaches such as viral-mediated shRNA knockdown. If the candidate activity is known to be regulated by enzymatic modifications (phosphorylation, cleavage, ubiquitination, etc.), their activation state will be verified during the time course of mSOD1 astrocyte-mediated motor neuron death. Then, the most promising candidates will be validated in vivo using knock out animal breeding or viral infection of motor neuron pools with shRNA in SOD1$^{G93A}$ mice. Together, these results will educate future studies on motor neuron cell death in ALS.

3.3.3. Are Developmental Cell Death Programs at Play in ALS?

A number of motor neuron cell death drivers were identified that are involved in neuronal patterning during development. What role, if any, could these factors play in ALS neurodegeneration? Whether these are a consequence of cellular stress or actual drivers of the motor neuron death would need to be explored. Another clue comes from Bax. Motor neurons undergo natural developmental death (≈40% loss) during development and patterning of the spinal cord (Hamburger, 1992). Whereas targeting necroptosis or apoptosis alone does not prevent the developmental cell death of motor neurons in the spinal cord as in mouse models (Kanungo et al., 2008; Oppenheim et al., 2001), there are no increases in the number of motor neurons observed,
and in Bax knockout mice the developmental death of motor neurons is prevented (White et al., 1998). Perhaps Bax is necessary for more than one mode of motor neuron cell death. In many cellular contexts, when apoptosis is blocked, necroptosis occurs as a backup mechanism. If Bax is a common element in both apoptosis and necroptosis, then its elimination would be sufficient. It is interesting that in both the context of development and in pathologic motor neuron cell death (Gould et al., 2006), Bax is the most potent inhibitor of motor neuron cell death, meaning that it is likely not involved in canonical apoptosis alone.

3.3.4. Exploiting the Motor Neuron Cell Death Signature in the Context of Necroptosis

These studies also raise the question: If knocking down NFkB1 phenocopies blocking RIPK1 kinase, what is the link between RIPK1 and NFkB1 regulation? By analyzing a motor neuron death transcriptome, Ikiz et al. (2015) identified potential drivers of motor neuron death. However, any response to ALS astrocyte-conditioned medium would be reflected in the signature, even a protective response rather than a driver of death. Experimental validation could eliminate those false leads. However, by first fully enriching the signature only for those elements that respond in a necroptosis-dependent manner (e.g., a “necroptosis-dependent gene expression signature”), this could be a more optimal approach. Given what we know about necroptosis in motor neurons and the differences that exist between our model of motor neuron death and classical apoptosis, and the limited models of the necroptosis transcriptome in the literature, such an analysis would greatly contribute both to our knowledge of motor neuron cell death and to identify new downstream mechanisms of necroptosis that have not been explored.

In order to elucidate the necroptosis-dependent gene expression signature of motor neuron
cell death, I would perform the same experiment as Ikiz et al. (2015) by exposing purified mouse ES-MN to ACM from mSOD1-expressing astrocytes, but with or without the inhibition of necroptosis via pharmacologic targeting of RIPK1 or genetic targeting of RIPK3. Then using a similar rationale, we could extract only those motor neuron death drivers enriched in the 3 day ACM signature that are reverted by inhibition of necroptosis.

The proposed study would identify motor neuron drivers of necroptosis and could be a useful approach to both answer the question as to the motor neuron-specific death program downstream of RIPK1 and tie together our data on Bax and NF-κB involvement. The proposed study could also generate new leads relevant for both motor neuron cell death and necroptosis mechanisms downstream of the necrosome. One attempt was made to generate the necroptosis-dependent gene expression signature of motor neuron cell death during my thesis, but too many samples failed to meet quality control criteria, and thus could not be used.

A second limitation to this work was using the whole brain interactome to study necroptosis. Unless this interactome was subjected to a necroptosis-relevant perturbation during its assembly, I cannot be sure that it captured the actual network of necroptosis. In addition, because the interactome was whole mouse brain and not specific to motor neurons, it might not be the most optimal interactome to fit the data in a cell-specific manner. Future studies would use a motor neuron-specific interactome if one becomes available. In addition, this motor neuron-specific interactome would ideally include exposure to a necroptosis-relevant perturbation during its assembly. The same RNA-seq data generated by Ikiz et al. (2015) could be rerun using the motor neuron-specific interactome, as well as the new RNA-seq data generated by the
aforementioned proposed experiment to generate a necroptosis-dependent gene expression signature of motor neuron cell death after exposure to ALS astrocytes.
Chapter 4: Immunoreagents to Study Necroptosis

4.1. Introduction

Necroptosis is a newly-recognized form of necrosis that is molecularly controlled (Degterev et al., 2005, 2008). Thus far it has been primarily studied in the field of immunology, in which it was discovered (Holler et al., 2000) by inducing cell death in in vitro systems with TNFα and using pan-caspase inhibitors such as zVAD-fmk, which has a high affinity for caspase-8 (Garcia-Calvo et al., 1998). Through these model systems, it was discovered that three key modular factors, namely RIPK1, RIPK3 and MLKL were driving the death process following a stereotypical cascade of events that can be summarized as follows. Upon death signal, RIPK1 becomes phosphorylated, which in turn recruits and phosphorylates RIPK3. RIPK3 then recruits and phosphorylates MLKL, which is believed the be the actual death effector of necroptosis by anchoring within the cell plasma membrane where it forms permeable pores leading to cell destruction (Cai et al., 2014; Chen et al., 2014; Su et al., 2014; Wang et al., 2014). While these deleterious events seem quite well documented, at least in vitro and in non-neuronal cells, I participated in a series of in vitro investigations, reported in the previous chapters, that provided compelling evidence supporting the notion that at least some of the core components of necroptosis may be conserved in neurons, and specifically motor neurons. While quite promising, a major limitation of the data presented in the previous chapters was that they all provided insight into a mode of cell death in an in vitro model system that was developed to emulate ALS in which the key cellular element, i.e., motor neurons, were immature (Ikiz et al., 2015; Re et al., 2014). Thus, a main outstanding question is whether necroptosis is a cell death mechanism by which mature
motor neurons die in vivo in the context of ALS. Relevant to this question is the recent study from Ito et al. (2016) in which the authors provided a host of immunoblot and immunocytochemical data consistent with necroptosis being involved in ALS pathogenesis. However, this study focused on the role of necroptosis in the activation of microglial inflammation, death of oligodendrocytes and ensuing axonopathy in two mouse models of ALS, but surprisingly failed to examine the potential role of this form of programmed necrosis in the demise of motor neurons, which has been the focus of my work. Accordingly, the goal of this chapter, built on the previous data generated by the Przedborski laboratory on neuronal necroptosis (see previous chapters) and on the Ito et al. study (2016), was to study the expression and status of RIPK1, RIPK3, and MLKL in a mouse model of ALS with emphasis on spinal cord and, more specifically, motor neurons.

4.2. Results

In Chapter 4, I focused my efforts on determining the status of the three key molecular determinants of necroptosis, at least as known in the immunological field, namely RIPK1, RIPK3 and MLKL. The studies were conducted primarily in transgenic SOD1<sup>G93A</sup> mice, which represent, up to now, the most extensively-validated and recognized mouse model of ALS. I began Chapter 4 by quantifying mRNAs for each of the aforementioned factors in the spinal cord of transgenic SOD1<sup>G93A</sup> mice. I then extended my work to their respective proteins to confirm that these core elements of necroptosis were expressed and detectable in the mouse spinal cord and to determine if the ALS-related disease process did alter their expression. I chose the spinal cord rather than the motor cortex, which also has motor neuron death in ALS, because lumbar spinal cord is the most well-studied and most affected area in the SOD1<sup>G93A</sup> mouse model.
4.2.1. Necroptosis mRNA Levels

Although expression of RIPK1, RIPK3 and MLKL in peripheral blood cells and other organ systems been well established (Newton and Manning, 2016), their expression in whole tissue of the CNS has been scarcely studied (Degterev et al., 2005; Ito et al., 2016; Kanno et al., 2015; Ofengeim et al., 2015). Indeed, recently, Fagerberg et al. (2014) used quantitative transcriptomics to analyze relative levels of protein coding transcripts across human tissues. While they did not test spinal cord, they did use brain, and indeed while present, mRNA levels of *RIPK1, RIPK3, and MLKL* were relatively low compared to other organs (Fagerberg et al., 2014). In addition, Wang et al. (2016) looked at relative levels of RIPK3 mRNA and protein in various wild-type mouse tissues, and found the lowest levels in brain tissue compared to other organs (Wang et al., 2016). Wu and collaborators (2013) surveyed MLKL protein levels in various mouse tissues, and found undetectable levels in several organs including the brain (Wu et al., 2013).

Thus, I wanted to determine whether I could detect *RIPK1, RIPK3, or MLKL* mRNA transcript levels in the spinal cord of wild-type mice and, if this was the case, compare levels with those assessed in symptomatic transgenic SOD1<sup>G93A</sup> mice to determine whether the mutant SOD1-induced disease process, which affects most severely the spinal cord in this model, might be associated with changes in the expression of these specific factors.

For these proposed experiments, I used RT-qPCR, which can detect as little as 1-10 copies of a given mRNA, so that if there were very low levels of transcripts and/or small magnitude of changes, in a small subset of cells in the spinal cord, I might be in an optimal position to detect them; I understood though that if I detected such small changes, the challenge would be to demonstrate their pathological significance.
To do these experiments, I extracted RNA from the lumbar spinal cords of symptomatic SOD1\textsuperscript{G93A} mice and age and strain-matched controls at 15 weeks. After generating cDNA by reverse transcription, I ran quantitative PCR using primers against RIPK1, RIPK3 and MLKL and used the transcript for the housekeeping GAPDH gene as a normalization control.

4.2.1.1. RIPK1, RIPK3, and MLKL mRNAs were detected in the mouse spinal cord

In whole spinal cord extracts from wild-type mice, the presence of RIPK1, RIPK3 and MLKL transcripts were consistently detected with average CT values of 26.05, 32.17, and 29.94, respectively, with average ΔCT of 9.08, 15.05, and 12.82 compared to GAPDH, respectively. Transcripts detected with CT values below 30 were generally accepted to be expressed, while those above 30 and especially above 35 were low abundance and easily affected by contamination of samples or reaction side-products. Although CT values for RIPK3 and MLKL were high, amplification curves showed product was being produced, and melting curves showed specificity of the primers to a single product (Figure 4.1, a-c). One confirmation that the CT value for RIPK3, although high, was reflecting specific signal was the use of the same primer against spinal cord sample from RIPK3KO mice with homozygous germline deletion of RIPK3 gene. Indeed, the CT value for RIPK3 in those mice was 35.26 and the ΔCT compared to GAPDH was 18.08, indicating a 6.6-fold reduction compared to wild-type mice and that at least part of the signal detected in wild-type spinal cord was specific for RIPK3. This was consistent with my findings in in vitro studies using spinal cord primary cultures; whereas CT values were consistently high for RIPK3 and MLKL, when using targeted shRNA lentiviral vectors to reduce the transcript I would consistently see higher CT values and relative fold reductions in RIPK3 and MLKL (data not shown). This led me to conclude that, although low, some specificity of signal was being detected.
For comparison, using the same RT-qPCR conditions and methods, expression of \textit{RIPK3} in wild-type spleen had a CT of 25.4, with a \( \Delta \text{CT} \) compared to \textit{GAPDH} of 6. Assuming \textit{GAPDH} was similarly expressed in spinal cord and spleen, this would suggest that \textit{RIPK3} was more than 500-fold less abundantly expressed in wild-type spinal cord compared to spleen. This was very approximate because a more optimal comparison would typically use multiple housekeeping genes and run the RT-qPCR in parallel “within run” for both tissues, if not an unbiased and absolute quantitative RNA-seq method. Nonetheless this approximate fold difference demonstrated that the levels of \textit{RIPK3} in wild-type mouse spinal cord were quite low and approaching the detection limits of the reaction.

For comparison of \textit{MLKL}, expression of \textit{MLKL} in L929 cells, which are known to express high levels of \textit{MLKL} and readily undergo necroptosis, had a CT of 24.3 for \textit{MLKL} and 6.98 \( \Delta \text{CT} \) compared to \textit{GAPDH}. This suggested, again by rough approximation, that in the spinal cord \textit{MLKL} was more than 50-fold less abundantly expressed.

\subsection{4.2.1.2. RIPK1, RIPK3, and MLKL mRNAs were increased in the spinal cord of symptomatic SOD1\textsuperscript{G93A} mice}

In whole spinal cord extracts from 12-week-old, early symptomatic transgenic SOD1\textsuperscript{G93A} mice, the presence of \textit{RIPK1}, \textit{RIPK3} and \textit{MLKL} transcripts were consistently detected with CT values of 31.51, 32.11, and 28.82, respectively, and \( \Delta \text{CT} \) compared to \textit{GAPDH} of 14.01, 14.61, and 11.32, respectively. Transcripts in age-matched controls were detected at CT of 31.34, 33.30, and 28.95 and \( \Delta \text{CT} \) compared to \textit{GAPDH} of 13.84, 11.45, and 15.80, respectively.
Male mice aged 12 weeks were perfused with ice-cold PBS and their spinal cords were dissected. RNA was extracted from the tissue and 1 µg of RNA was used per reaction to reverse-transcribe into cDNA for qPCR. Individual lines represent individual reactions. Graphs show aggregate data from three replicate reactions of all samples used in the 12 weeks analysis (NTg n=2, SOD1<sup>G93A</sup> n=3). a-b. Real-time quantitative qPCR amplification curve (x-axis= time in cycles, y-axis= fluorescence) using SYBR-green detection methods and primers against RIPK1, RIPK3, MLKL and GAPDH. c. Melting curve analysis shows a single peak for RIPK1, RIPK3 and MLKL primed reactions [x = temperature, y= decrease in fluorescence/time (top) or fluorescence (bottom)]

These values indicate that RIPK1 mRNA levels were not changed the spinal cord of 12-week-old symptomatic SOD1<sup>G93A</sup> mice as compared to age-matched wild-type controls (two-tailed
Student’s t-test, df=4, p= 0.14, Figure 4.2, a). Likewise, \textit{MLKL} mRNA levels were not changed (two-tailed Student’s t-test, df=4, p=0.8, Figure 4.2, c). As for \textit{RIPK}3 mRNA levels, they were 2.15-fold higher in the spinal cord of symptomatic SOD\textsubscript{1G93A} mice as compared to age-matched wild-type controls (two-tailed Student’s t-test, df=4, p= 0.4, Figure 4.2, b).

In whole spinal cord extracts from 15-week-old symptomatic transgenic SOD\textsubscript{1G93A} mice, the presence of \textit{RIPK1}, \textit{RIPK}3 and \textit{MLKL} transcripts were consistently detected with CT values of 25.68, 31.73, and 29.72, respectively, and $\Delta$CT compared to \textit{GAPDH} of 8.45, 14.31, and 12.29, respectively. Age-matched controls were detected at CT of 27.76, 32.1675 and 29.935 and $\Delta$CT compared to \textit{GAPDH} of 9.085, 15.05 and 12.82, respectively.

These values indicated that \textit{RIPK1} mRNA levels were 1.55-fold greater in the spinal cord of symptomatic SOD\textsubscript{1G93A} mice as compared to age-matched wild-type controls (Figure 4.2, d). Likewise, \textit{RIPK}3 mRNA levels were 1.67-fold greater in the spinal cord of symptomatic SOD\textsubscript{1G93A} mice as compared to age-matched wild-type controls (Figure 4.2, e). As for \textit{MLKL} mRNA levels, they were 1.45-fold greater in the spinal cord of symptomatic SOD\textsubscript{1G93A} mice as compared to age-matched wild-type controls (Figure 4.2, f). Unfortunately, I did not have enough wild-type control tissue at this time point to have n=3 for each condition, so I could not determine statistical analysis. However, I could conclude that the RT-qPCR results suggested that spinal cords of symptomatic SOD\textsubscript{1G93A} mice showed either unchanged or higher transcript levels for the three core components of necroptosis, not lower. Such modest increases in mRNA transcript may not be biologically relevant. I explore this question in part II and III to look at whether the protein products of these three genes were in fact increased as a result.
Figure 4.2 Increases in mRNA of Core Necroptosis Components \textit{RIPK1, RIP3, and MLKL} in Symptomatic Mouse SOD1\textsuperscript{G93A} Spinal Cord

Male mice at 12 (n=3 NTg, n=3 SOD1\textsuperscript{G93A}) or 15 (n=2 NTg, n=3 SOD1\textsuperscript{G93A}) weeks postnatal age were perfused with ice-cold PBS and lumbar spinal cords were dissected out and used for RNA extraction, preparation of cDNA, and qPCR. Using primers against \textit{RIPK1, RIPK3, and MLKL} and housekeeping gene \textit{GAPDH}, the results were the following: In the SOD1\textsuperscript{G93A} spinal cord at 12 weeks, there was no change in levels of \textit{RIPK1} (a, p= 0.14) or \textit{MLKL} (c, p=0.80) mRNA, but there was a 2.15-fold increase of \textit{RIPK3} (b, p=0.04). In the SOD1\textsuperscript{G93A} spinal cord at 15 weeks (symptomatic) \textbf{d}, \textit{RIPK1} mRNA was increased 1.55-fold, \textbf{e}, \textit{RIPK3} mRNA was increased 1.67-fold, and \textbf{f}, \textit{MLKL} mRNA increased 1.45-fold. Two-tailed Student's t-tests were performed.)
Therefore, in this section I showed that \textit{RIPK1}, \textit{RIPK3}, and \textit{MLKL} transcript were detected in mouse spinal cord, however the levels of \textit{RIPK3} and \textit{MLKL} transcript were quite low. I also showed modest increases for \textit{RIPK1}, \textit{RIPK3}, and \textit{MLKL} transcript in the spinal cord of 15 weeks symptomatic SOD1\textsuperscript{G93A} mice, but only \textit{RIPK3} was increased at an earlier time point of 12 weeks.

\textbf{4.2.2. Validation of Antibodies Against Necroptosis Proteins for Immunoblot}

Now that I determined that \textit{RIPK1}, \textit{RIPK3} and \textit{MLKL} mRNAs were expressed in mouse spinal cord, even if they were at low levels, I decided to examine whether their respective products could be detected in the mouse spinal cord by using available immunoreagents that have been used in several published studies (Ito et al., 2016; Miao and Degterev, 2009; Ofengeim et al., 2015; Orozco et al., 2014).

However, in reviewing these different papers, I was struck by the fact that few, even as part of supplementary data, included any validation of these different antibodies for western blot, except in the case of RIPK1 (Moujalled et al., 2013; Orozco et al., 2014), but not immunocytochemistry, leaving me with only the material provided by the commercial vendors. Though I selected the antibodies based on extensive use in publications, it thus appeared essential to me to formally validate the selected antibodies (see Table 4.1) for their use in both immunoblot and immunohisto-/immunocyto-chemistry prior to embarking on the actual studies in ALS mice.
Before validation, and because I had in hand commercially-available lysate for use as positive controls for western blotting, I ran some of the antibodies against this lysate in order to ensure I was using optimal detection methods to reveal my bands of interest, and more importantly, to determine whether the antibodies cleanly detected a single band or detected many bands of various weights, as can occur when antibodies recognize proteins other than those against which they were raised. I had mouse NIH3T3 lysate, which is known to express RIPK1 and MLKL, but not RIPK3 (He et al., 2011; Sun et al., 2012). I ran two MLKL and two RIPK1 antibodies against the NIH3T3 lysate (Figure 4.3, a-d). I also ran RIPK1 antibodies against human Jurkat cell lysate (Figure 4.3, c-d), as these two antibodies were raised against a fragment of human RIPK1 protein (Table 4.1), and although there is sequence similarity between mouse and human RIPK1, I wanted to be certain that if I did not see a band in mouse, it was not because the antibody failed to recognize mouse RIPK1. I did not use MLKL antibodies or RIPK3 antibodies against the Jurkat lysate because both were raised against mouse protein (Table 4.1), the product sheets did not indicate their use in human, and in all future experiments I would be using these antibodies against mouse tissue. The results were the following: both MLKL antibodies detected just one prominent band at

### Table 4.1 Antibodies Against Necroptosis Core Elements Selected for Validation

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antigen Region</th>
<th>Antibody Clone</th>
<th>Species Specificity</th>
<th>Company</th>
<th>Product #</th>
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<tr>
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<td>S10458</td>
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<td>H, M</td>
<td>BD Biosciences</td>
<td>3493</td>
<td>Y</td>
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<tr>
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<td>Human RIP peptide near Leu190</td>
<td>D94C12</td>
<td>H, M</td>
<td>Cell Signaling</td>
<td>15628</td>
<td>Y</td>
</tr>
<tr>
<td>RIPK3</td>
<td>Mouse RIPK3 peptide near His 411</td>
<td>D8J3L</td>
<td>M</td>
<td>Cell Signaling</td>
<td></td>
<td></td>
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<td>Rabbit Polyclonal</td>
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<td>AP14272b</td>
<td>Y</td>
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<tr>
<td>MLKL</td>
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<td>Rabbit Polyclonal</td>
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<td>Y</td>
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<tr>
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<td>EPR8515(2)</td>
<td>M</td>
<td>Abcam</td>
<td>196436</td>
<td>N</td>
</tr>
</tbody>
</table>

*Legend: “Validation (in house)” = for western blotting

**but extensively validated in literature

** but likely the same as AP14272b
the expected molecular weight of MLKL at 54 kD (Figure 4.3, a-b), and both RIPK1 antibodies detected just one prominent band at 74 (mouse NIH3T3) or 76 (human Jurkat) corresponding to the predicted molecular weight of RIPK1 protein (Figure 4.3, c-d). Assuming these bands were specific, I concluded I was using optimal detection methods for both RIPK1 and MLKL proteins via western blot. In addition, these four antibodies did not recognize other proteins in the control lysate (Figure 4.3, a-d). Therefore, I confidently continued with further validations using negative controls to ensure specificity of the prominent band for each antibody.

4.2.2.1. Validation of RIPK1-Specific Antibody for Western Blot

I chose two RIPK1 antibodies to validate. One was chosen based on its validation in the literature for western blot (He et al., 2009; Orozco et al., 2014) and the other because of its previous use in mouse spinal cord samples for western blot (Ito et al., 2016). However, it had not been validated with positive and negative controls. In order to validate RIPK1 antibodies for immunoblot, I decided to silence RIPK1 in E12.5 primary spinal culture from a wild-type mouse using a lentiviral vector expressing a validated RIPK1 shRNA. Cells were isolated, infected by spinoculation as I have done before (Re et al., 2014), and cultured. Four days post-infection, RNA was extracted and used for RT-qPCR. With this approach, I found that this silencing procedure produced a 78% knockdown of RIPK1 transcript (Figure 4.4, c), which I thought should be sufficient to detect a change in RIPK1 protein. From the same cells, protein lysate was extracted and used for immunoblot, using two different antibodies, which both revealed one prominent band corresponding to ~74 kDa in both cells infected with RIPK1 shRNA and non-mammalian control shRNA (Figure 4.4, a-b, 4.4, d-e). In the immunoblot loaded with proteins from the former, I noticed a 51% reduction of the band that corresponded to ~74 kDa using the first antibody (Figure
4.4 a, b), and 66% reduction using the second antibody (Figure 4.4, a-b) which is consistent with the molecular weight of RIPK1 protein.

**Figure 4.3 Using Control Cell Lysates as Positive Controls to Test Antibodies Against RIPK1 and MLKL**

**a-d:** 20 μg per lane of protein extract from mouse NIH3T3 cells or human Jurkat cells (whole cell lysates in sample buffer purchased from Abgent, Inc. San Diego, CA) was separated by SDS-PAGE and transferred to nitrocellulose membrane. After blocking with blocking buffer for fluorescent western blotting (Rockland Immunochemicals, Limerick, PA) membranes were probed overnight at 4°C in blocking buffer with the indicated primary antibodies against MLKL and RIPK1. The next day, membranes were washed 3x with 0.1% Pbs and probed with infrared probe-conjugated secondary antibodies, washed 3x with 0.1% Pbs, and were imaged by the Li-cro Odyssey Imaging System.
4.2.2.2. Validation of RIPK3-Specific Antibody for Western blot

Thus, I followed a similar approach to identify and validate a RIPK3 antibody that was recently used to detect RIPK3 protein in mouse spinal cord for immunoblot (Ito et al., 2016). Given the low levels of RIPK3 transcript in the spinal cord, as I saw in part I, I turned my attention to the spleen, a known organ with high levels of RIPK3 mRNA and protein. Using spleen from adult wild-type or mice with homozygous germline deletion of RIPK3 (RIPK3KO), which are known to not
express RIPK3 (Newton et al., 2004), I tested the specificity of the antibody for western blot. Some of these RIPK3KO mice were perfused with ice-cold phosphate buffered saline (PBS) to remove the blood prior to extracting RNA from the spleen, and this material was used to confirm by RT-qPCR the lack of RIPK3 transcript (Figure 4.5, c).

Upon running immunoblots with spleen proteins from both wild-type and RIPK3KO mice using ECL detection methods, I observed two prominent bands, one at 47 kD and one at 55 kD, and a smear between 47 and 55 kD (Figure 4.5, a). The prominent band around 55 kDa, which is consistent with RIPK3 known molecular weight, was markedly less visible in RIPK3KO using ECL detection methods (Figure 4.5, a) and even absent when using Li-cor detection (Figure 4.5, d). It is possible that the lower molecular weight bands seen below the 55 kD band were degradation or cleavage products, though the prominent 47 kD band was not consistent with the predicted (~36 kD) or observed (~39 kD) reported C-terminal RIPK3 cleavage product generated by caspase-8 at Asp328 of the RHIM domain (Feng et al., 2007).
Figure 4.5 A RIPK3 Antibody was Validated for Western Blot by Comparing Spleen Protein Extract from Wild-type or Mice with Germline Deletion in RIPK3

Adult mice, either wild-type or with germline deletion of RIPK3 [RIPK3KO obtained from Genentech (Newton et al., 2004)], were perfused with ice-cold PBS and the spleen was dissected. Protein was extracted and prepared for western blot under denaturing conditions, and RNA was extracted and prepared for qPCR. a. Western blot using RIPK3 primary antibody (Cell Signaling, #15828) and ECL detection methods showed a marked reduction in a 55 kD band in RIPK3KO spleen tissue quantified in b. c. qPCR using primers against RIPK3 and housekeeping gene GAPDH showed a 100% reduction in RIPK3 mRNA d. Western blot using RIPK3 primary antibody (Cell Signaling, #15828) and the Li-cor Odyssey Imaging System showed undetectable levels in the 55 kD band in RIPK3KO spleen tissue.
4.2.2.3. Validation of MLKL-specific Antibody for Western Blot

Using a similar rationale, I identified two MLKL antibodies in the literature, one because of its use for multiple western blots in a recent publication showing a very clear high-intensity band (Yoon et al., 2016), and the other because it was used recently to detect MLKL in mouse spinal cord (Ito et al., 2016). Both MLKL antibodies that I identified had shown a clean band against mouse NIH3T3 lysate at expected molecular weight 54 kD (Figure 4.3). However, because both antibodies were rabbit polyclonal against mouse C-terminal MLKL (Table 4.1) and one was a product of Sigma, I suspected that they could be in fact the same, meaning generated from the same animal, because they are polyclonal, as Sigma is a purchasing partner of Abgent’s antibodies. Although I could not get a clear answer from Sigma, a representative at Abgent confirmed that these two products are likely the same based on product notes. Therefore, I continued with the validation of the antibody from Abgent, since the it likely originated from there, and the product page listed the specifics about the region of the protein used as an immunogen (Table 4.1), so I was more certain about what region of the protein I was detecting.

Here, I knocked down MLKL transcript in mouse L929 cells, a necroptosis-sensitive fibrosarcoma cell line known to express high levels of MLKL protein, by using a lentiviral vector expressing shRNA against MLKL. As above, after 4 days, RNA was extracted and used for RT-qPCR for MLKL transcript. Although I found that my silencing strategy gave only a 65% reduction of MLKL transcript (Figure 4.6, c), I decided to pursue my validation with this reagent as I thought that this moderate reduction in MLKL mRNA may suffice to have a meaningful impact on the expression of its product. Consistent with my assumption, I found that there was a 48% reduction in the intensity of a band localized on the immunoblot at around 54 kDa (Figure 4.6, a-b), which is consistent with the known MLKL molecular weight. No other bands were observed on the
immunoblot, providing encouragement that this antibody may be suitable for the planned experiments.

---

**MLKL Antibody Validation in L929 Cells**

- **a**. L929 cells
  - CTL RNAi
  - MLKL 54 kD
  - β-actin 43 kD

- **b**. Western blot quantification
  - Relative MLKL protein levels

- **c**. qPCR quantification
  - Relative MLKL RNA levels

**P-MLKL Antibody Validation in L929 Cells**

- **d**. L929 cells
  - CTL TSZ 1hr
  - P-MLKL 54 kD
  - β-actin 43 kD

- **e**. Western blot quantification
  - Relative P-MLKL protein levels

---

**Figure 4.6 An MLKL Antibody and a P-MLKL Antibody were Validated for Western Blot Using Mouse L929 Cultures**

- **a-c**. Mouse L929 cell line cultures were plated and 24 hours later lentivirus containing shRNA against MLKL (Sigma Mission # 360819) was applied for 4 days at MOI 100, after which RNA and protein was extracted. **a**. Western blot using MLKL primary antibody (Abgent, Inc.) showed a 48% reduction in band intensity in a 54 kD band quantified in **b, c**. qPCR quantification of MLKL RNAi showed 65% reduction in MLKL mRNA.
- **d-e**. L929 cells were treated with human recombinant TNF-α (30 ng/mL), smac mimetic (TL-32711) (100 nM) and zVAD-fmk (20 µM) (TSZ) for 1 hour and protein was extracted. **d**. Western blot showed a 6-fold increase in a 54 kD band in the TSZ-treated cells quantified in **e**.

---

**4.2.2.4. Validation of phospho-MLKL-Specific Antibody for Western Blot**

Lastly, I validated the only commercially-available mouse MLKL phospho-serine 345 antibody (Table 4.1), a site that is phosphorylated during necroptosis signaling. Thus, to induce necroptosis
in mouse L929 cells, I exposed these cells to TNFα, smac mimetic (TL-32711), and caspase inhibitor zVAD-fmk treatment (TSZ), a treatment known to induce necroptosis in L929 cells (He et al., 2009) the kinetics of which were recently further characterized by Ros et al. (Ros et al., 2017). I predicted that the phosphorylated MLKL band would be more intense in the immunoblot following necroptosis induction compared to control cells. First, to confirm necroptosis induction using this method, cells were live-imaged at 8 hr post-treatment using Hoescht (blue) to count the number of cells present in the dish as well as propidium iodide (PI) (red) to quantify the number of dying cells that have lost plasma membrane integrity and therefore would readily take up the dye (Figure 4.7). After 8 hr, no live cells remained on the plate in the TSZ-treated group compared to control (Figure 4.7). Necroptosis was confirmed as the cell death mechanism by using necrostatin-1, as indeed, at 8 hr post co-treatment with necrostatin-1 and TSZ, the number of Hoescht positive nuclei were close to that of the control condition, although there were some PI positive cells (Figure 4.7). Because all of the cells were dead in the TSZ alone condition, in order to extract protein, I used an earlier time point, i.e., 1 hr after TSZ addition. Indeed, this is similar to the kinetics recently reported by Ros et al. (Ros et al., 2017) that showed necroptosis induction at 1-hour post TSZ treatment in L929 cells. L929 control cell protein extract and 1-hr TSZ-treated were used for immunoblot. A band at 54 kDa consistent with phospho-MLKL protein molecular weight was observed to be increased 6-fold in TSZ-treated cells (Figure 4.6, d-e). Other less prominent bands were observed on the blot as well, but none were increased in the TSZ condition (data not shown). These results support the usefulness of this immuno-reagent even if given the other bands detected, its value for immunohisto/immunocytochemistry is uncertain. Therefore, I successfully validated antibodies against mouse RIPK1, RIPK3, MLKL, and phosphorylated MLKL for use in western blot.
Figure 4.7 Necroptosis Induction in L929 Cells

L929 cells were treated with human recombinant TNF-α (30 ng/mL), smac mimetic (TL-32711) (100 nM) and zVAD-fmk (20 µM) (TSZ) with or without necrostatin-1 (20 µM) for 8 hours and incubated with propidium iodide (red) and hoescht (blue) for 15 minutes at 37°C, washed, and live imaged using fluorescent microscopy.

4.2.3. Signs of Core Necroptosis Factors Activation in the Spinal Cord of SOD1G93A Mice

Using the immunoblot-validated antibodies discussed above, I wanted to now see whether I could find evidence of necroptosis protein activation in the spinal cord of symptomatic SOD1G93A mice. A reasonable assumption would be that if necroptosis was ongoing, protein levels of the core necroptosis proteins RIPK1, RIPK3, and MLKL would increase as a result of their stabilization due to their assembly in the necroptosome complex (Li et al., 2012) and based on what others have reported (Ito et al., 2016; Ofengeim et al., 2015). In order to better assess activation of necroptosis,
rather than simple upregulation of protein levels, I also looked for signs of increased MLKL phosphorylation using the phosphoserine 345 specific antibody validated in Part I. For the following studies, lumbar spinal cord protein extract from adult symptomatic SOD1\textsuperscript{G93A} (15 weeks) and age-matched, background strain-matched controls were used to assess necroptosis protein levels and activation by immunoblot.

4.2.3.1. RIPK1 Levels Increase in the Spinal Cord of Symptomatic SOD1\textsuperscript{G93A} Mice

Consistently, I detected the validated RIPK1 band in the wild-type spinal cord extracts (Figure 4.8). Interestingly, there was a two-fold increase in RIPK1 band intensity in the symptomatic SOD1\textsuperscript{G93A} mice compared to wild-type mice (two-tailed Student’s t-test, df=4, p=0.0037) (Figure 4.8, a-b).

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**Figure 4.8 RIPK1 Protein Levels are Increased in Symptomatic SOD1\textsuperscript{G93A} Mouse Spinal Cord**

Male B6/SJL mice at 15 weeks postnatal age (n=3 NTg, n=3 SOD1\textsuperscript{G93A}) were perfused with ice-cold PBS and lumbar spinal cords were dissected out and protein was extracted in RIPA lysis buffer (150 mM NaCl, 25 mM Tris pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS, protease inhibitor cocktail and phosphatase inhibitor (Sigma, cOmplete™, PhosSTOP™)). a. western blot using RIPK1 antibody (BD Biosciences, Clone 38/RIP) and b. quantification showed a two-fold increase in intensity of the RIPK1 band at 74 kD in the SOD1\textsuperscript{G93A} spinal cord lysate (two-tailed Student’s t-test, df=4, p=0.0037) 60 ug of protein per lane of protein was loaded.
4.2.3.2. RIPK3 is Undetectable in the Mouse Spinal Cord

In contrast to RIPK1, I did not detect RIPK3 protein in wild-type nor in symptomatic SOD1\textsuperscript{G93A} mice even by loading 50-60 µg of protein per lane and by using the most sensitive technical available to me, i.e., ECL detection method (Figure 4.9, a-c). I identified the most prominent band observed, at 52 kD, as nonspecific as it is much lower than any predicted molecular weight for RIPK3 and did not run at the same level as the positive control (Figure 4.9, a-c). The nonspecific nature of this band was confirmed by using spinal cord extract from RIPK3KO mice (Figure 4.9, b-c).

I also know that this negative result was not attributable to my protein preparation as I tried a variety of lysing conditions, ranging from commercially-available tissue lysis buffer, RIPA buffer, to urea-containing lysis buffers, and based on published protocols (Afonso et al., 2015; Ito et al., 2016; Miao and Degterev, 2009) used for the study of necroptosis. Using lysis buffer containing 6 M urea, the most stringent that I tried, again detected RIPK1 in the spinal cord of both wild-type and SOD1\textsuperscript{G93A} mice (Figure 4.10, a). Because I did not observe the increase that I saw at 15 weeks, I re-affirmed these findings using RIPA lysis buffer to extract protein at 12 weeks, and found similar results, with no quantified increase in RIPK1 protein (Figure 4.10, b-c). As this tissue was taken at 12 weeks, and I did not see an increase in RIPK1, it suggested that the increase I observe at 15 weeks might be concomitant with disease progression. These results correlated with observed mRNA expression, in that in part 4.2.1.2. I observed an increase in \textit{RIPK1} mRNA at 15 weeks but not 12 weeks. This suggested that the observed increase in RIPK1 protein could in part be due to transcriptional upregulation. More protein samples, at 12 weeks, and at additional time points during disease progression, would be needed to confirm this, but it was interesting to note that the increase in RIPK1 I observed at 15 weeks could be a result of a more advanced
pathology at that age. Nonetheless, this further validated that the 6 M urea-containing lysates I prepared were suitable to detect the proteins of interest. When I ran the RIPK3 antibody I validated in part 4.2.2.2. using the sensitive ECL method and 50 ug of protein per lane against the wild-type and 12 weeks SOD1\textsuperscript{G93A} samples, I saw a prominent band around 52 kD, and a fainter band at ~70 kD, neither of which disappear in lysate from RIPK3KO mice, so I concluded that both were nonspecific (Figure 4.10, d).

As the last recourse, I could try to enhance the RIPK3 signal if its spinal cord content is so low by subjecting the extracts first to an immunoprecipitation step and then run the immunoblot. However, I did not pursue this option as I was doubtful that even if positive, such a low amount of RIPK3 protein could be sufficient to have a meaningful pathological significance, and notably, immunoprecipitation is not as amenable to quantifying differences in protein levels between individual samples. Instead, I thought that a more fruitful approach would be to cross the RIPK3KO mice with the transgenic mSOD1 mice and see if the absence of RIPK3 would have a disease-modifying effect; this experiment is presented in Chapter 5.
Figure 4.9. RIPK3, MLKL, and P-MLKL Protein Levels Were Undetectable in Symptomatic SOD1<sup>G93A</sup> Mouse Spinal Cord

a, d-e: Male B6/SJL mice at 15 weeks postnatal age (n=3 NTg, n=3 SOD1<sup>G93A</sup>) were perfused with ice-cold PBS and lumbar spinal cords were dissected out. Protein was extracted in RIPA lysis buffer [150 mM NaCl, 25 mM Tris pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS, protease inhibitor cocktail and phosphatase inhibitor (Sigma, cOmplete™, PhosSTOP™)]. a. Western blot using RIPK3 antibody (Cell Sig. Clone D8J3L) showed no signal at 55 kD compared to wild-type spleen control. d. Western blot using MLKL antibody (Abgent) showed no signal at 55 kD compared to NIH3T3 cell lysate control. e. Western blot using P-MLKL antibody (Abcam, #196436) showed no signal at 55 kD compared to TSZ-treated L929 cell lysate control. b-c: Protein from male C57Bl/6 mice at 19 weeks age (n=1 SOD1<sup>G93A</sup>, n=1 RIPK3KO/SOD1<sup>G93A</sup>) was extracted as in a. b. Western blot using RIPK3 antibody (Cell Sig. Clone D8J3L) showed no signal at 55 kD compared to wild-type spleen control and nonspecific band at 50 kD did not change in intensity in RIPK3KO/SOD1<sup>G93A</sup>. c. Higher exposure of b. showed similar results. 60 ug of protein per lane was loaded of the spinal cord lysate, 25 ug of protein was loaded for the spleen tissue lysate, NIH3T3 lysate, and TSZ-treated L929 lysate.
Figure 4.10. RIPK3, MLKL, and P-MLKL Protein Levels Were Undetectable in Early Symptomatic SOD1<sup>G93A</sup> Mouse Spinal Cord

Male B6/SJL mice at 12 weeks postnatal age (n=1 NTg, n=1 SOD1<sup>G93A</sup>) were perfused with ice-cold PBS and lumbar spinal cords were dissected out. Protein was extracted in urea lysis buffer [6 M urea, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EGTA, 50 mM NaF, 10 mM b-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM benzamidine, 1 mM PMSF, 5 mM N-ethylmaleimide, 1 mM DTT, protease inhibitor cocktail and phosphatase inhibitor (Sigma, cOmplete™, PhosSTOP™)] according to Ito et al. (2016). a-b, Western blot of spinal cord (a) RIPA (b) urea lysate using the indicated RIPK1 antibodies and c, quantification of b. d-e, Western blot of spinal cord urea lysate using the indicated antibodies and RIPK3KO (RIPK3 negative control) and NIH3T3 lysate (MLKL positive control). 50 µg of protein per lane was loaded of the spinal cord lysate 25 µg per lane of NIH3T3 lysate.
4.2.3.3. MLKL Was Likewise Undetectable in the Mouse Spinal Cord

I encountered a similar situation for MLKL as for RIPK3 in that I did not detect MLKL protein in wild-type nor symptomatic SOD1\textsuperscript{G93A} mice, even when loading 50-60 ug of protein per lane. Lysate from NIH3T3 cells, which are known to express high levels of MLKL, was run as an in-run positive control and showed high signal at 54 kDA using the same antibody validated in Part I (Figure 4.9, d). No other bands of different sizes were detected on the blot. (Figure 4.9, d). This again, as for RIPK3, raised the same question as to whether the protein levels of MLKL in the spinal cord were very low and under the detection limit of the validated immunoreagent.

Again, I tried running using several other published protein preparations, the most stringent of which was 6 M urea-containing lysis buffer, using sensitive ECL detection methods, and using both MLKL antibodies that showed me a clean band at the expected molecular weight in the NIH3T3 lysate (Figure 4.10, e). Again, I saw a nonspecific band at 52 kD that was much less prominent and ran below the very prominent 54 kD band in the NIH3T3 lysate (Figure 4.10, e). In fact, using the urea lysis buffer and ECL detection methods, for whatever reason, I often saw this nonspecific band at 52 kD, as well as a faint band at ~70kD, both of which were also observed in the RIPK1 and RIPK3 immunoblot from the same lysate (Figure 4.10, a, d, e). This further led me to conclude that these two bands were not specific.

4.2.3.4. Phosphorylated MLKL Levels Were Undetectable in the Mouse Spinal Cord

Although in the absence of MLKL protein detection cited above, I could have omitted the study of phospho-MLKL in our mouse spinal cord extracts. However, I thought it may still be worth exploring as the phospho-MLKL antibody may be more sensitive than the c-terminal MLKL antibody and that detection of a small trace of phospho-MLKL would still be highly significant.
I did not detect phosphorylated MLKL protein in wild-type nor symptomatic SOD1\textsuperscript{G93A} mice (Figure 4.9, e). A band that ran lower than 52 kD, at around 50 kD, was observed but concluded to be nonspecific as it ran slightly lower than the positive control band in the lysate from necroptosis-induced L929 cells (Figure 4.9, e). This was not surprising because I also could not detect total MLKL in the spinal cord as even a faint band. The antibody against total MLKL did not detect a region that contains the phosphorylation site (see Table 4.1) so the likelihood that there would be inhibition of this antibody to detect a subset of phosphorylated MLKL was low, so if there was a subset of phosphorylated MLKL it could theoretically also be detected in the total MLKL blot and it was not.

In this section, using the reagents I validated for western blotting, RIPK1 showed a modest but significant increase in protein levels in the spinal cord of symptomatic SOD1\textsuperscript{G93A} mice compared to controls. However, RIPK3 and MLKL levels were undetectable by western blotting in the same samples, even loading high levels of protein per lane and using high stringency buffers.

4.2.4. Detecting Necroptosis Proteins in Human ALS Patient Samples

In light of the increase in RIPK1 in the spinal cord of SOD1\textsuperscript{G93A}, I was curious to see if similar RIPK1 alteration could be detected in postmortem tissue from sporadic and SOD1 ALS patients. While I did not have readily-available patient spinal cord tissue, I was kindly provided with brain homogenate from Broadmann’s area 4, which contains the motor cortex, from patients with sporadic ALS, SOD1 ALS, and age-matched control patients. The samples were a gift from and previously prepared as described by Erin Conlon (Conlon et al., 2016) in collaboration with the Shneider Lab at Columbia University. I prepared protein extracts under denaturing conditions
using equivalent amounts of sample. Patient history, when available, is described for each sample in Table 4.2.

### Table 4.2 ALS Patient Samples History

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<td>F</td>
<td>diffuse weakness, proximal involvement</td>
</tr>
<tr>
<td>sALS #8</td>
<td>Sporadic ALS</td>
<td>~55</td>
<td>M</td>
<td>Lower extremity onset, used high dose steroids (prednisone, decadron, testosterone)</td>
</tr>
<tr>
<td>sALS #9</td>
<td>Sporadic ALS</td>
<td>~70</td>
<td>F</td>
<td>Bulbar onset</td>
</tr>
<tr>
<td>sALS #10</td>
<td>Sporadic ALS</td>
<td>69</td>
<td>F</td>
<td>Fast, ~20 month disease course, bulbar onset</td>
</tr>
<tr>
<td>fALS #11</td>
<td>SOD1 mutation, L144F</td>
<td>52</td>
<td>F</td>
<td>Limb onset, 4 year disease course</td>
</tr>
<tr>
<td>fALS #12</td>
<td>SOD1 mutation (unknown)</td>
<td>31</td>
<td>F</td>
<td>2 year disease course</td>
</tr>
</tbody>
</table>

#### 4.2.4.1. RIPK1 in Human Samples

Using two different antibodies against RIPK1, one validated in part II that also recognizes human RIPK1 (recall Jurkat extracts), and one that has been extensively validated in the literature for human RIPK1 (Luan et al., 2015)(Miao and Degterev, 2009), I looked for changes in RIPK1
protein between the samples. Both antibodies detected a band at 76 kD, consistent with RIPK1, and a slightly higher and much fainter band (not shown) at 80-85 kD, the identity of which is unknown (Figure 4.11, a,b). While I found consistent results between the two antibodies (Figure 4.11, a-c), what I found was that levels of RIPK1 were variable across all samples, with no clear trend among patients. For example, there was no correlation with higher levels in the ALS patient samples compared to controls, as high and low expressers were seen in both pools (Figure 4.11, a-c). I also did not see an obvious correlation with patient history; those patients with higher levels of RIPK1 did not necessarily have a more aggressive disease (Table 4.2, Figure 4.11 a-c).

However, because these samples were taken postmortem, and because RIPK1 has roles in necrosis and necroptosis in response to ischemia, it is possible that the samples had baseline levels of necrosis and or necroptosis, from the exposure to ischemic conditions in the tissue at the time of death (Degterev et al., 2005; Vieira et al., 2014). Therefore, even in the control patients the protein profile may not reflect the status of the tissue before losing blood supply. Postmortem samples may not be ideal to detect changes between patients in these particular proteins involved in cell death for this reason. Interestingly, it was recently found that significant transcriptional upregulation can occur postmortem, especially in the brain (Pozhitkov et al., 2017), and that many of the transcripts upregulated during the first hour encode proteins that are involved in cell death and inflammation.

The RIPK3 and MLKL antibodies which I validated in part II were not optimal for use against the human proteins as they recognized mouse epitopes (Table 4.1). In the future, I could obtain and validate human RIPK3 and human MLKL-specific antibodies to test their levels in these samples. What would be even better would be to obtain a phosphorylation-specific human MLKL antibody, which is commercially available and has been used by other groups, as this would

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reflect an activation state rather than simply upregulation of the protein. However, given the
documented caveats of using postmortem samples, and the lack of detection of these proteins
in the mouse tissue, this was not explored in the current work.

![Image of Western blots and gel analysis](image)

**Figure 4.11. Measuring RIPK1 Levels in Postmortem Brain Cortex (Broadmann’s Area 4)**

Protein lysate was prepared as described in (Conlon et al., 2016) from pulverized human brain from motor cortex
(Broadmann’s area 4) in lysis buffer (0.1 M MES (pH 7), 1 mM EDTA, 0.5 mM MgSO₄, 1 M sucrose, 50 mM N-
éthylmaleimide, 1 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF and 10 µg/ml each of aprotinin, leupeptin and pepstatin) at
18 µl/mg of tissue. ~4 µL of soluble homogenate was loaded per lane. Samples lanes are labeled as according to Table
4.2. **a-b** Western blot on samples was performed using the indicated RIPK1 antibodies and **c**, quantification of relative
band intensity between samples.
I did, however have an antibody against human phosphorylated-RIPK3, kindly provided by Jiahuai Han at Xiamen University, China, whose group previously validated the antibody (Chen et al., 2013) against phosphorylated threonine 231/serine 232, a site which promotes MLKL interaction and necroptosis (Chen et al., 2013, 2015; McQuade et al., 2013). Using this antibody, I did get a band at 55 kD corresponding to the molecular weight of phosphorylated RIPK3, but the levels of that band, if specific, were the same across all samples (Figure 4.12).

Figure 4.12. Measuring p-RIPK3 Levels in Postmortem Brain Cortex (Broadmann’s Area 4)

Protein lysate was prepared as described in (Conlon et al., 2016) from pulverized human brain from motor cortex (Broadmann’s area 4) in lysis buffer (0.1 M MES (pH 7), 1 mM EDTA, 0.5 mM MgSO₄, 1 M sucrose, 50 mM N-ethylmaleimide, 1 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF and 10 μg/ml each of aprotinin, leupeptin and pepstatin) at 18 μl/mg of tissue. ~4 μL of soluble homogenate was loaded per lane. Samples lanes are labeled as according to Table 4.2. a-b Western blot on samples was performed using anti-human phospho-RIPK3 (threonine 231/serine 232) antibodies generated by (Chen et al., 2013) and c. quantification of relative band intensity between samples.
4.2.5. No Antibodies Against Necroptosis Were yet Identified as Suitable for Immunostaining

I was very curious to see which cell types were responsible for the mRNA and protein expression patterns in the mouse wild-type and symptomatic SOD1\(^{G93A}\) spinal cord. Given the increase on whole spinal cord tissue I observed in RIPK1 protein in symptomatic SOD1\(^{G93A}\), I was curious to see, as per my original hypothesis from the conclusions of Chapters 2 and 3 in vitro studies, whether necroptosis proteins were indeed increased in motor neurons in vivo in the context of motor neuron degeneration. I could not exclude the possibility that the increased RIPK1 content in the whole spinal cord tissue of symptomatic transgenic SOD1\(^{G93A}\) mice I observed did not reflect changes in other cells, such as glial cells rather than in motor neurons.

I focused my studies on the expression of RIPK1, in particular, because it is the only one of the three core factors I was able to detect in mouse spinal cord tissue at reasonable levels by western blot. Thus, I first sought to confirm whether the two antibodies validated for western blotting were indeed suitable for detection using immunohistochemistry. Then, I would use the antibodies to stain tissue sections of spinal cord from wild-type and symptomatic SOD1\(^{G93A}\) mice.

4.2.5.1. Western Blot-Validated RIPK1 Antibodies Did Not Show Specific Signal for Immunostaining in Same Cell Culture used for Western Blot and qPCR

First, in order to identify antibodies that may be suitable for immunostaining against RIPK1, cells from the same spinal cord primary culture that was used to validate RIPK1 antibody specificity on western blot (Figure 4.4) were also fixed on coverslips and stained with the two antibodies against RIPK1 that showed specificity via western blot in Figure 4.4. The product sheets available for these antibodies indicated they could be suitable for use in immunostaining. While there was some faint signal present for both antibodies that required relatively high exposure via fluorescent
microscopy to capture (in the seconds rather than milliseconds), by taking pictures at the same exposure time, there was no change in this signal between control cells and cells with 50% reduction in the western blot band, 78% reduction in RIPK1 transcript (Figure 4.13, a-b). I confirmed that the cells were prepared properly for immunostaining by co-staining with anti-GFP antibody, which labels motor neurons in this culture as they are from a motor neuron promoter (HB9) GFP reporter line. Representative images are shown of motor neurons stained with anti-GFP antibody in the same culture (Figure 4.13, c). Therefore, I did not conclude that these antibodies could be used with confidence, and so I did not identify a suitable antibody to detect RIPK1 protein via immunostaining. Since I did not have the advantage of having a negative control for RIPK1 in vivo in the spinal cord, as germline deletion of RIPK1 is early postnatal lethal, even if I stained spinal cord, I would not know with certainty that the staining was specific based on this experiment.

4.2.5.2. No RIPK3-Specific Antibody Was Identified as Suitable for Immunohistochemistry to Detect RIPK3 in the Spinal Cord of Wild-type and Symptomatic SOD1G93A mice

Even though I did not detect RIPK3 in whole spinal cord protein lysate, it was possible that a small number of cells expressed RIPK3 and therefore did not enrich enough in the pooled sample to detect. Therefore, I was very interested to see if I could detect RIPK3 in the spinal cord of animals, especially in symptomatic SOD1G93A mice. As for identifying a suitable antibody, I had the advantage of having mice with homozygous germline deletion for RIPK3, which I further confirmed do not express RIPK3 in previous sections II and III, which served as a negative control. I also had the advantage of a recent study demonstrating an increase in RIPK3 immunostaining in
Figure 4.13 RIPK1 Antibodies Did Not Show Specific Signal for Immunostaining in Mouse Primary Spinal Cord Cultures

Primary neuronal cultures from mouse embryo E12.5 were prepared infected with lentivirus containing shRNA against RIPK1 by spinoculation exactly as previously described (Re et al., 2014) and plated for 4 days in culture, after which RIPK1 knockdown was validated by western blot and qPCR (Figure 4.3), and cells were fixed on coverslips and a-b immunostained for RIPK1 using the indicated antibodies or c. GFP, which labels motor neurons in this culture (GFP reporter under HB9 promoter). No change in RIPK1 immunostaining was observed by fluorescence microscopy.
the mouse spinal cord in a spinal cord injury model (Kanno et al., 2015). This study indicated no or little RIPK3 immunostaining in wild-type mouse spinal cord, but an increase in signal following spinal cord hemi-transection compared to sham surgery. They reference a rabbit RIPK3 antibody from Sigma, and, I concluded that this was #2283 as it was the only mouse-specific RIPK3 antibody offered by Sigma that was suggested for use for immunostaining. Sigma purchases this antibody clone from ProSci, and so I used the ProSci #2283 product for my experiment. Based on the results generated by Kanno et al. (2015), I assumed that I might not detect RIPK3 in adult wild-type mouse spinal cord using this antibody. Therefore, I decided to compare levels of RIPK3 immunostaining in the spinal cord of symptomatic SOD1G93A mice either on a wild-type background or a RIPK3KO background. My assumption was that, if RIPK3 protein did increase in this model in a subset of cells, as suggested by the modest increase in RIPK3 transcript I measured, I would be in optimal conditions to detect the signal, and should see no signal in the RIPK3KO tissue. I also did compare wild-type mice to RIPK3KO mice, with the assumption that if I did see a signal in wild-type mice it might not be specific for RIPK3 based on Kanno et al. study not measuring RIPK3 signal in wild-type animals. Using the same antibody and similar tissue preparation and staining protocol reported by Kanno et al. (2015), I did see faint signal in all four of these conditions, mostly labeling neurons based on their morphology and nuclei, but there was no change in this signal between wild-type background or RIPK3KO background, in neither the non-transgenic or SOD1G93A-expressing animals, indicating that this staining I observed was not specific for RIPK3 (Figure 4.14, a-h).

Based on these results, and identifying off-target signal using this antibody, I had the idea to run an immunoblot with this new antibody using NIH3T3 lysate, which as I mentioned in section II, should not express RIPK3 (He et al., 2011; Sun et al., 2012). Indeed, while the validated RIPK3
antibody from section II (Cell Sig. D8J3L), showed no signal, the new RIPK3 ProSci #2283 antibody used by Kanno et al. (2015) showed many prominent bands in the same lysate and using the same immunoblotting conditions, which should be nonspecific due to the reported lack of RIPK3 in this lysate (Figure 4.15, a). This further indicated to me that this antibody was not suitable for western blotting, nor was it suitable for immunostaining due to many off-target interactions.

Unfortunately, the RIPK3 antibody I validated for western blot (Cell Sig. D8J3L) was not indicated on the product sheet as suitable for immunostaining, so I did not try this, although I could in a future study. One additional experiment I could do to be absolutely certain of my results would be to induce the spinal cord injury in mice as Kanno et al. (2015) have done, to have an optimal positive control. Nonetheless, I concluded so far that I have not yet identified an antibody that shows specific immunostaining for RIPK3 in vivo.
Figure 4.14 No RIPK3-Specific Immunohistochemistry Labeling in the Ventral Horn

Male B6/SJL mice at 17 weeks postnatal age, NTg, SOD1<sup>G93A</sup>, RIPK3KO/NTg, RIPK3KO/ SOD1<sup>G93A</sup> perfused with PBS followed by 4% paraformaldehyde and lumbar spinal cord was dissected and post-fixed overnight at 4°C. Samples were embedded in 10% sucrose/gelatin and flash frozen and 20 micron sections were cut at the cryostat and mounted on slides. Sections were immunostained with RIPK3 antibody (ProSci #2283) and choline acetyltransferase (ChAT) antibody (Millipore, AB144P).
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Figure 4.15 RIPK3 Antibody Recognizes Many Nonspecific Bands on Western Blot

a. 20 ug per lane of protein extract from mouse NIH3T3 cells (whole cell lysate in sample buffer purchased from Abgent, Inc. San Diego, CA) was separated by SDS-PAGE and transferred to nitrocellulose membrane. After blocking with blocking buffer for fluorescent western blotting (Rockland Immunochemicals, Limerick, PA) membranes were probed overnight at 4°C in blocking buffer with the indicated primary antibodies against the indicated RIPK3 antibodies. The next day membranes were washed 3x with 0.1% Pbsl and probed with infrared probe-conjugated secondary antibodies, washed 3x with 0.1% Pbsl and membranes were imaged by the Li-cor Odyssey Imaging System.

4.3. Discussion

In this chapter, I performed an extensive validation of immunoreagents raised against core factors of necroptosis. This task was prompted by the realization that a growing number of publications are reporting quite convincing immunoblot and immunohistochemical and immunocytochemical
data in a variety of pathological situations including non-neurological such as steatohepatitis (Afonso et al., 2015b) and neurological such as multiple sclerosis (Ofengeim et al., 2015) and more recently ALS (Ito et al., 2016). In light of these studies, it was quite tantalizing to try to use these reagents to study necroptosis in ALS, but by focusing at the protein and cellular levels in both animal models and even postmortem samples from ALS patients. While Ito et al. (2016) proposed a role for necroptosis in ALS by showing activation in oligodendrocytes and microglia (Ito et al., 2016), surprisingly there was no report on whether necroptosis is activated in motor neurons to drive cell death, as my previous in vitro data suggests.

Here, I showed that RIPK1, RIPK3, and MLKL transcripts were detected in mouse spinal cord, however the levels of RIPK3 and MLKL transcript were quite low. I also showed modest but significant increases for RIPK1, RIPK3, and an increasing trend in MLKL transcript in the spinal cord of symptomatic SOD1<sup>G93A</sup> mice, the site of motor neuron neurodegeneration in these animals.

I then validated immunoreagents to study levels of RIPK1, RIPK3, and MLKL in the spinal cord of symptomatic SOD1<sup>G93A</sup> mice. While I found suitable reagents and conditions for immunoblotting of RIPK1, RIPK3, MLKL and necroptosis-specific phosphorylated MLKL, I did not yet identify suitable immunoreagents to study necroptosis at the cellular level in vivo in mouse tissue, despite the reports of others.

Using the reagents, I validated for western blotting, RIPK1 showed a modest but significant increase in protein levels in the spinal cord of symptomatic SOD1<sup>G93A</sup> mice compared to controls. However, RIPK3 and MLKL levels were undetectable by western blotting in the same samples, even loading high levels of protein per lane and using high stringency buffers. I cannot exclude that RIPK3 and MLKL were indeed present but below the level of detection by western blotting. This was not entirely surprising, as in the case for RIPK3, I estimated that based on my qPCR
results the mRNA was enriched in spinal cord at 500-fold less than in the spleen, where it is abundantly expressed. Likewise, for MLKL, the mRNA was expressed 50-fold less than in L929 cells where it is abundantly expressed. If RIPK3 and MLKL proteins were indeed present, they were likely expressed in a small number of cells that did not enrich the whole spinal cord extract enough to detect on western blotting, and/or were expressed at low levels throughout the tissue.

My finding that RIPK3 and MLKL transcript and protein levels were low in the mouse spinal cord in part support the findings of Kanno et al. (2015) whereby there was no detection of RIPK3 protein in the spinal cord of adult wild-type mice by immuno labeling. These findings were also supported by Wang et al. (Wang et al., 2016), who by studying relative levels of RIPK3 mRNA and protein in various wild-type mouse tissues, found the lowest levels in brain tissue compared to other organs. In addition, Wu et al. (Wu et al., 2013) surveyed MLKL protein levels in various mouse tissues, and found undetectable levels in several organs including the brain (Wu et al., 2013). This directly calls into question the abundant signal observed by Ito et al. (2016) for RIPK3, MLKL, and phospho-MLKL in the spinal cord of both wild-type and early-symptomatic SOD1<sup>G93A</sup> mice.

My results led me to question whether, given such low levels of RIPK3 and MLKL protein, necroptosis could play a meaningful role in the mechanism of motor neuron death in SOD1<sup>G93A</sup> mice. However, this conclusion is fraught with assumptions. First, assuming that necroptosis does indeed play a role in motor neuron death in ALS, one important point to make is that motor neuron death is not synchronous in this model. The degeneration and death occurs over a period of time, so that at any one given time, only a subset of motor neurons in the spinal cord are in the same window of cell death. Second, it is only an assumption, based on what others have shown, that levels of necroptosis proteins would increase based on necroptosis activation, rather than signaling
through post-translational modifications alone, e.g., phosphorylation-mediated signal transduction. Third, there is evidence that increasing protein levels of RIPK3 and MLKL by overexpression alone promotes necroptosis signaling, regardless of RIPK1 activation, due to homodimerization/self-associated activation (Dondelinger et al., 2014; Geserick et al., 2015; Moujalled et al., 2013; Zhao et al., 2012), meaning that there would be an advantage for cells to keep baseline levels of RIPK3 and MLKL protein low to minimize aberrant necroptosis activation, especially in post-mitotic neurons that are not regularly turning as in other tissues. Supporting this notion is the work of Kanno et al. (2015) showing undetectable RIPK3 protein at baseline in the spinal cord but rapidly increasing in protein expression following spinal cord injury specific to the injury site.

Still, I am intrigued by the increase in RIPK1 mRNA and protein levels I observed. As RIPK1 has roles in pro-survival, apoptotic, and necroptotic signaling (Festjens et al., 2007; Newton, 2015; Newton and Manning, 2016), I would be curious to identify the specific role RIPK1 could be playing here given the significant increase in spinal cord in symptomatic SOD1<sup>G93A</sup> mice, and, if suitable immunoreagents become available, to study which cell types show this increase in RIPK1. I would also be curious to observe whether the increasing RIPK1 fraction contained post-translational modifications, such as ubiquitination and phosphorylation, that could give me a clue as to which downstream mechanism of RIPK1 could be resulting from the increase. A recent study rather elegantly demonstrated the coordinated phosphorylation and site-specific ubiquitination events during RIPK1-mediated necroptosis signaling (de Almagro et al., 2016), and by mass spectrometry identified K115 as a necroptosis-specific ubiquitination site on human RIPK1. De Almagro et al. also suggested this site, which is evolutionarily-conserved, may be functionally conserved in mouse RIPK1 protein. Therefore, I could use a similar approach to identify whether
this site is preferentially ubiquitinated in RIPK1 from the in spinal cord of symptomatic SOD1\textsuperscript{G93A} mice compared to wild-type mice.
Chapter 5: Genetic Deletion of RIPK3 as Disease-Modifying Strategy in ALS

5.1 Introduction

As mentioned in the previous chapter, we have collected a series of compelling data supporting the notion that necroptosis can kill motor neurons in in vitro models of both familial and sporadic ALS (Re et al., 2014). To further support the involvement of necroptosis in ALS, I tried to use a series of published immunoreagents to examine by immunoblot the tissue content and by immunohistochemistry the cellular expression of the three core factors of necroptosis, namely RIPK1, RIPK3 and MLKL. However, since using the immunoreagents, at least in my hands, did not prove useful to study necroptosis in vivo, I thought of an alternative strategy and perhaps one that is more mechanistic. I thus posited that one way to assess the contribution, if any, of the necroptosis pathway to the ALS-like pathology in the SOD1\textsuperscript{G93A} mice might be to target obligate mediators of necroptosis and assess any measurable improvement of the ALS-like phenotype. The way I tested this idea was through genetic means by crossing mice with homozygous germline deletion of RIPK3 (RIPK3\textsuperscript{-/-}, kindly provided by Genentech) with transgenic SOD1\textsuperscript{G93A} mice. I had three reasons to choose this model. The first was that RIPK3\textsuperscript{-/-} mice had already been shown to be resistant to a variety of necroptosis stimuli. The second was that while RIPK3\textsuperscript{-/-} mice are healthy, RIPK1\textsuperscript{-/-} die soon after birth. The third, related to this and previously discussed in other chapters, while RIPK3 has few non-redundant roles outside of necroptosis, RIPK1 as a scaffold is a critical mediator NF-κB pro-survival and inflammation pathways independent of its role in necroptosis.
Newton et al. (2004) generated these RIPK1−/− mice by using a targeting vector to remove the first 3 exons of RIPK3 gene, which encode amino acids 1-158 of the protein, and confirmed that RIPK3 protein was not produced in various cells and organs. Whereas RIPK1-deficient mice die soon after birth, RIPK3−/− mice survive to adulthood without health or fertility defects, ripk3−/− homozygotes occur in litters at the expected Mendelian frequency, and histological analysis does not show defects in major organs (Newton et al., 2004). In my hands, I observed the RIPK3−/− mice also survived, bred and gained weight in a normal manner (Figure 5.1). In chapters 2 and 4, I further confirmed that I did not detect RIPK3 transcript in various tissues relevant to the present study such as embryonic and adult spinal cord, in adult spleen, nor RIPK3 protein in adult spleen of these RIPK3−/− mice.

![Figure 5.1 RIPK3−/− Mice Had Normal Weight Gain](image)

**Figure 5.1 RIPK3−/− Mice Had Normal Weight Gain**

Mice (C57Bl/6N, n=1 for each condition m=male, f=female, RIPK3WT= wild-type littersmates, RIPK3KO=RIPK3−/− littersmates) were weighed at the given time points and weights (in grams) were plotted over time. Slopes of the lines are similar showing weight gain over time is equivalent in the genotypes, even though there can be variation in individual mouse weight.
5.2 Results

5.2.1 Male, But Not Female, Transgenic SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{−/−} Mice Showed Delayed Onset and Prolonged Survival

5.2.1.1 Onset in SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{−/−} mice

Mice of each genotype were weighed 2-3 times per week, and onset was defined in this case as the age (postnatal day) at which a mouse lost 10\% of its peak weight. Given that much of a mouse’s weight is determined by muscle mass, this correlated with loss of muscle mass related to the denervation and atrophy in this model. Using this parameter, I found no difference overall between SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{−/−} mice (median=155) and SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{+/+} mice (median=152) (Figure 5.2, a, Log-rank Mantel-Cox test, p= 0.108).

Likewise, when I stratified the data per sex, age at onset was not different between transgenic SOD1\textsuperscript{G93A} male (median=152) and female (median=155) mice (Figure 5.2, b, Log-rank Mantel-Cox test, P=0.168). However, SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{−/−} male mice showed a significantly longer time to end-stage onset (median=155) as compared to SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{+/+} male (median=152) (Figure 5.2, c, Log-rank Mantel-Cox test, P= 0.0050). Even though the medians were similar, the curve comparison through statistical testing indicated a shift in onset. Strikingly, this genotypic difference was only seen in males as it was not observed in female mice, which had no significantly different age at end-stage onset (Figure 5.2, d, Log-rank Mantel-Cox test, P=0.8356), even though the medians were quite different (SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{+/+} female = 155, SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{−/−} female = 147.5).
Lastly, improvement in the male mice did not extend onset past the point of the typical female transgenic SOD1\textsuperscript{G93A} mice, with the medians both at 155 (Log-rank Mantel-Cox test, P=0.281).

\textbf{Onset (>10\% Weight Loss)}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure52.png}
\caption{Onset in SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{−/−} Mice}
\end{figure}

SOD1\textsuperscript{G93A} mice of each genotype were weighed 2-3 times per week, and onset was defined as the age (postnatal day) at which a mouse lost 10\% of its weight from its peak weight during the study. \textbf{a.} Onset was not different overall between SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{−/−} mice (median=155) and SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{+/+} mice (median=152) (n=8-9 mice per condition). (Log-rank Mantel-Cox test, p=0.108) \textbf{b.} Age at onset was not different between transgenic SOD1\textsuperscript{G93A} male (median=152) and female (median=155) mice (Log-rank Mantel-Cox test, P=0.168). \textbf{c.} Onset in SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{−/−} male mice showed a significantly longer time to onset (median=155) as compared to SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{+/+} males (median=152) (Log-rank Mantel-Cox test, p=0.0050) \textbf{d.} Onset in SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{−/−} female mice (median=147.5) was not significantly different age than SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{+/+} females (median=155) (Log-rank Mantel-Cox test, P=0.8356) (for b-d, n=4-5 mice per condition).
5.2.1.2 Survival of SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> Mice

At end stage, muscle wasting and weakness had progressed and spread rapidly, and mice were thus sacrificed humanely when they were no longer able to right themselves from supine position after 30 seconds. In each mouse, survival time was recorded as postnatal day from birth to loss of righting ability. Again, there was no overall difference in survival between transgenic SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> mice (median=168.5) and SOD1<sup>G93A</sup>/RIPK3<sup>+/+</sup> mice (median=162.5) (Figure 5.3, a, Log-rank Mantel-Cox test, p= 0.0678).

![Survival of SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> Mice](image)

**Figure 5.3. Survival of SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> Mice**

Survival age was recorded as postnatal day from birth to loss of righting ability, within 30 seconds from being placed in a supine position. SOD1<sup>G93A</sup> mice of each genotype. a. No difference in survival was found between transgenic SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> mice (median=168.5) and SOD1<sup>G93A</sup>/RIPK3<sup>+/+</sup> mice (median=162.5) (Log-rank Mantel-Cox test, p= 0.0678). b. Transgenic SOD1<sup>G93A</sup> female mice did survive longer than male mice (medians=170, 157, respectively) (Log-rank Mantel-Cox test, P= 0.0256). c. SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> male mice survived significantly longer than SOD1<sup>G93A</sup>/RIPK3<sup>+/+</sup> male mice (medians=178, 157, respectively) (Log-rank Mantel-Cox test, P=0.0170). d. Survival was not significantly different between the SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> and the SOD1<sup>G93A</sup>/RIPK3<sup>+/+</sup> female mice (medians=163, 170 respectively) (Log-rank Mantel-Cox test, P=0.7205). n=10-12 mice per group in a, and 5-6 mice per group in b-d.
When I stratified the data by sex, transgenic SOD1\textsuperscript{G93A} female mice survived longer than male mice (medians=170, 157, respectively) (Figure 5.3, b, Log-rank Mantel-Cox test, P=0.0256). Furthermore, I found that SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{-/-} male mice survived significantly longer than SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{+/+} male mice (medians=178, 157, respectively) (Figure 5.3, c, Log-rank Mantel-Cox test, P=0.0170). Conversely, likewise to the age of onset, survival was not significantly different between the SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{-/-} and the SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{+/+} female mice (medians=163, 170 respectively) (Figure 5.3, d, Log-rank Mantel-Cox test, P=0.7205).

Similar to onset of end stage, the improvement of survival of transgenic SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{-/-} male mice did not exceed the survival of the typical female transgenic SOD1\textsuperscript{G93A} mice (medians=178, 170, respectively) (Log-rank Mantel-Cox test, P=0.1070).

5.2.2 Sex-Specific Improvements in Onset and Survival Did Not Correlate with Functional Motor Tests

A visual representation of functional motor tests is presented in Figure 5.4, and results are described below.

5.2.2.1 Grip Strength Measured by Inverted Grid Test

Starting at 9 weeks of age, mice were tested once per week by recording the maximum inverted hanging time on a grid, using all four limbs, up to 60 seconds as best of three trials, a test of grip strength (Oliván et al., 2015).
Figure 5.4. Visual Representations of Functional Motor Tests

Top. Left: Mouse performing inverted grid test Right: mouse performing a loaded grid test Bottom. Series of grids presented to mice for loaded grid test.
SOD1^{G93A}/RIPK3^{-/-} mice did not hold longer on average compared to SOD1^{G93A}/RIPK3^{+/+} mice (Figures 5.5, a, 5.7, a). Student’s t-test for significance was performed for each time point, and p values were recorded (Figure 5.7, a), none were significant. Non-transgenic mice not carrying the SOD1^{G93A} mutant transgene were also tested for both RIPK3 genotypes and always held the entire 60 seconds, with no variation, data not shown. There was no difference in sexes on the inverted grid test (Figure 5.6 a,c-d, Figure 5.8, a, e, f).

5.2.2.1 Grip Strength Measured by Loaded Grid Test

Starting at 6 weeks of age, mice were tested twice per week by recording their weight and maximum time holding a series of weighted grids, using all four limbs, up to 30 seconds as best of three trials, a test that was established by Barnéoud et al. (1997) to detect early changes in grip strength in ALS mouse models. Briefly, the mouse was suspended by the tail and allowed to grip a series of grids with increasing weights (10, 20, 30, 40g) and a behavioral score was calculated as follows: score=\[\sum(t_w \times W)/\text{body weight}\] where \(t_w\) corresponds to the maximum time the mouse was able to hold the grid at weight \(W\). Body weight is the weight of the animal at the time of the test. A maximum period of 30 seconds was allowed for each weight and best of three trials was recorded.
Figure 5.5. Functional Motor Tests on SOD1<sup>G93A</sup> RIPK3<sup>−/−</sup> Mice

**a.** Mice were tested once per week starting at 9 weeks of age and maximum hold time was recorded up to 60 seconds. SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> mice did not perform better on inverted grid test compared to SOD1<sup>G93A</sup>/RIPK3<sup>+/+</sup> mice. Student’s t-test was performed for each time point and found in Figure 5.7 a. **b.** Starting at 6 weeks of age, mice were tested twice per week by recording their weight and maximum time holding a series of weighted grids, using all four limbs, up to 30 seconds as best of three trials, to calculate an integrated score (see text). Student’s t-test was performed for each time point and found in Figure 5.7 a. n=12-15 mice per group SOD1<sup>G93A</sup>, 5-6 mice per group NTg.
Mice were separated by genotype and sex for grip strength analysis. a-b Loaded grid test a. Male and female SOD1<sup>G93A</sup> mice inverted grid test b. Male and female SOD1<sup>G93A</sup> mice loaded grid test c-d. Inverted grid test c. Male SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> and SOD1<sup>G93A</sup>/RIPK3<sup>+/+</sup> mice d. Female SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> and SOD1<sup>G93A</sup>/RIPK3<sup>+/+</sup> mice e-f. Loaded grid test e. Male SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> and SOD1<sup>G93A</sup>/RIPK3<sup>+/+</sup> mice f. Female SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> and SOD1<sup>G93A</sup>/RIPK3<sup>+/+</sup> mice. n=6-8 mice per group.

Figure 5.6. Functional Motor tests on Tests on SOD1G93ARIPK3−/− Mice by Sex
SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{-/-} mice did not hold grids longer on average compared to SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{+/-} mice (Figure 5.5, b). Student’s t-test for significance was performed for each time point, and p values were recorded (Figure 5.7, b). Non-transgenic mice not carrying the SOD1\textsuperscript{G93A} mutant transgene were also tested for both RIPK3 genotypes for score comparison (Figure 5.5, b). No differences were observed (Figure 5.7, c).

Grip strength tests were re-analyzed separating male and female mice. There was no improvement between SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{-/-} and SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{+/-} mice of the same sex (Figure 5.6, b, e-f, Figure 5.8, b, c-d). The female SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{+/-} did perform better on the loaded grid test at some time points compared to female SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{-/-} mice (Figure 5.6, f, 5.8, d). In my study, SOD1\textsuperscript{G93A} females performed better on the loaded grid test than males at some time points during symptom onset (Figure 5.6, b, 5.8, b).
**Figure 5.7. Statistical Results of Functional Motor Tests in RIPK3−/− Mice**

a-c. Multiple two-tailed Student’s t-tests were run comparing the indicated groups at each time point. There was no statistical difference in at any time point in SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> and SOD1<sup>G93A</sup>/RIPK3<sup>+/−</sup> mice overall (n=12-15 mice per group) in a. inverted grid or b. loaded grid or between c. non-transgenic RIPK3<sup>−/−</sup> and RIPK3<sup>+/−</sup> mice.

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Figure 5.8. Statistical Results of Functional Motor Tests in RIPK3<sup>−/−</sup> mice by Sex

Multiple two-tailed Student’s t-tests were run comparing the indicated groups at each time point. There was no difference at any time point between male and female SOD1<sup>G93A</sup> mice in **a**, inverted grid, but **b**, females performed better at some time points (red). There was no difference in at any time point between male SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> and SOD1<sup>G93A</sup>/RIPK3<sup>+/+</sup> mice for **c**, loaded grid or **d**, inverted grid. **d**, Female SOD1<sup>G93A</sup>/RIPK3<sup>+/+</sup> performed better at some time points (red) compared to SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> at loaded grid but not **f**, loaded grid. NA= scores not available. n=6-8 mice per group.
5.2.3. Sex-Specific Improvements in Onset and Survival Did Not Correlate with Morphological Markers Either

5.2.3.1. Quantification of Motor Neuron Loss at End Stage

Motor neuron number was quantified as number of cytoplasmic choline acetyltransferase (ChAT)-positive neurons per hemisection in the lateral ventral horn of a 20-micron section of lumbar spinal cord from the lumbar L4 and lumbar L5 segment (Figure 5.9). Figure 5.10, a, shows an example of ChAT staining of motor neurons in the ventral horn of the lumbar spinal cord of an adult wild-type mouse. A neuron would be counted if the nucleus was also present on the section, identified by using a DAPI counterstain (not shown).

Figure 5.9. Schematic Representation of Lumbar Section Used for Motor Neuron Counting

Motor neuron number was quantified as number of cytoplasmic ChAT-positive neurons per hemisection in the lateral ventral horn of a 20-micron section of lumbar spinal cord from the lumbar L4 and lumbar L5 segment (blue) identified by the roots under a microscope. **Left.** End-stage SOD1<sup>G93A</sup> mouse **Right.** ChAT+ staining in the ventral horn of a 20-micron section of lumbar spinal cord.
**Figure 5.10. Example of Immunostaining Used for Quantifications**

**a.** Example of ChAT immunostaining of motor neurons in the ventral horn of the lumbar spinal cord of an adult wild-type mouse. 20-micron thick section was imaged by confocal microscopy and maximum intensity projection of the z-plane was used. **b.** Example of completely innervated neuromuscular junction staining by colocalization of BTX (Alexa 594 conjugated alpha-bungarotoxin, red) and VACHT (anti-voltage gated acetylcholine transporter, green) in the tibialis anterior muscle of a wild-type adult mouse. Scale bar 20 micron.
Motor neuron number was measured at end-stage postnatal day 140 (P140). There was no difference between the number of motor neurons present in P140 SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> mice compared to SOD1<sup>G93A</sup>/RIPK3<sup>+/+</sup> mice (Two-tailed Student’s t-test, p= 0.4221) (Figure 5.11, a).

There were no inherent differences in motor neuron number at P140 between non-transgenic RIPK3<sup>−/−</sup> and RIPK3<sup>+/+</sup> mice (Two-tailed Student’s t-test, P=0.2192) (Figure 5.11, a).

Representative images show ChAT-positive immunostaining in sections used for motor neuron counting from each genotype (Figure 5.12).

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**Figure 5.11. Quantification of Pathology in SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> Mice**

a. Motor neuron number was quantified at end-stage postnatal day 140 (P140). There was no difference between the number of motor neurons present in P140 SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> mice compared to SOD1<sup>G93A</sup>/RIPK3<sup>+/+</sup> mice (Two-tailed Student’s t-test, p= 0.4221) There were no inherent differences in motor neuron number at P140 between non-transgenic RIPK3<sup>−/−</sup> and RIPK3<sup>+/+</sup> mice (Two-tailed Student’s t-test, P=0.2192). b. At least 100 neuromuscular junctions on 20-micron tibialis anterior sections were stained and counted for each mouse in symptomatic mice at postnatal day 120. There was no difference between number of innervated or denervated neuromuscular junctions between SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> mice and SOD1<sup>G93A</sup>/RIPK3<sup>+/+</sup> mice (Two-tailed Student’s t-test, p=0.6650 innervated, P=0.4847 denervated). There was no difference in partially-innervated neuromuscular junctions (Two-tailed Student’s t-test, p=0.5636). n= 3 biological replicates per condition. Error bars represent SEM of the mean.
5.2.3.2. Quantification of innervation at the neuromuscular junction in symptomatic mice

Sections of tibialis anterior (20-micron thickness) were cut and neuromuscular junctions were immunostained for presynaptic voltage gated acetylcholine transporter (VaChT) and post-synaptic using fluorescence conjugated alpha-bungarotoxin (BTX). Neuromuscular junction innervation was quantified by identifying BTX-positive NMJs (red) and determining the extent of colocalization with VaChT (green) (Figure 5.10, b). Full innervation was defined as greater than 70% overlap of BTX and VaChT, partial at 30-70% overlap, and denervation at 0-30% overlap.

![Figure 5.10 Representative Images of Motor Neuron Counting](image)

Lumbar ventral horns show ChAT+ immunostaining in representative images used for motor neuron quantification. **Top** left: NTg/RIPK3+/+ right: NTg/RIPK3−/− **Bottom** left: SOD1G93A/RIPK3+/+ right: SOD1G93A/RIPK3−/− 20-µm sections were imaged on fluorescent microscope at 10X magnification.
Representative images show neuromuscular junction staining (NMJ) by colocalization of BTX (Alexa 594 conjugated alpha-bungarotoxin, red, post-synaptic) and VACHT (anti-voltage gated acetylcholine transporter, green, presynaptic) in the tibialis anterior muscle of left: SOD1G93A/RIPK3+/+ right: SOD1G93A/RIPK3−/− mice at symptomatic postnatal day P120. 20-micron sections were labeled and imaged on a fluorescent microscope at 10X magnification. White arrows indicate neuromuscular junctions that would be counted based on BTX labeling (being within the plane of the image). In each image, 2 arrows are showing a denervated NMJ.

At least 100 neuromuscular junctions on 20-micron tibialis anterior sections were stained and counted for each mouse in symptomatic mice at postnatal day 120. There was no difference between number of innervated or denervated neuromuscular junctions between SOD1G93A/RIPK3+/+ mice and SOD1G93A/RIPK3−/− mice (Two-tailed Student’s t-test, p=0.6650 innervated, p=0.4847 denervated) (Figure 5.11, b). There was no difference in partially-innervated neuromuscular junctions (Two-tailed Student’s t-test, p=0.5636) (Figure 5.11, b). Representative images show
images neuromuscular junction staining in tibialis anterior muscle sections used for quantification (Figure 5.13).

5.3. Discussion

5.3.1. Sex-Specific Improvements in Male SOD1$^{G93A}$ Mice Lacking RIPK3

My study was not powered to detect differences by sex, as both males and females were pooled per genotype to reach the desirable number of mice (N=12-15 mice per group). I therefore ran post-hoc analyses by separating sexes to determine whether there were differences biasing the study. Surprisingly, although sex differences in the C57Bl/6J background SOD1$^{G93A}$ have not been previously reported (Heiman-Patterson et al., 2005), my study did identify a difference in survival between male and female SOD1$^{G93A}$ mice, although not in onset or grip strength.

Typically, sex differences have been seen on the B6/SJL background in SOD1$^{G93A}$ mice, in that females survive longer (Heiman-Patterson et al., 2005).

Although both the SOD1$^{G93A}$ mice and RIPK3$^{-/-}$ mice used in this study were both on the C57Bl/6 background, there have been strain differences affecting phenotypes observed between C57Bl/6J (that the SOD1$^{G93A}$ mice originate from) and C57Bl/6N (that the RIPK3$^{-/-}$ mice originate from)(Newton et al., 2004; Simon et al., 2013). Since the mice in my study were of a mixed background (C57Bl/6N and C57Bl/6J) they could not be compared exactly to data on C57Bl/6J SOD1$^{G93A}$ mice that did not show sex differences in survival. However, my data do suggest that careful observation of both sexes during mouse studies of ALS is critical to the interpretation of the results.
Regardless of the reason for the difference, in light of my initial findings, I separated sexes and re-analyzed SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> mice compared to SOD1<sup>G93A</sup>/RIPK3<sup>+/+</sup> of the same sex. After performing this gender segregation, there were modest improvements in disease onset and survival in male SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> mice that were not observed in female mice. However, these results did not correlate with improvements in grip strength. Furthermore, in light of these results all pathology analysis of tissue, including motor neuron number and innervation of neuromuscular junction were performed in male mice. I did not see improvements in SOD1<sup>G93A</sup>/RIPK3<sup>−/−</sup> mice pathology either, at any of the tested time points.

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**Figure 5.14. Average Weight of Mouse Cohorts Over Time in SOD1<sup>G93A</sup> Study**

An average of the weights over time of all cohorts of SOD1<sup>G93A</sup> mice in the study at the indicated genotypes and sex (n= 10-15 mice per condition). Red arrow points to an earlier decline in weight as a cohort of male SOD1<sup>G93A</sup> mice.
Another approach to interpreting the onset data is to analyze average weight of each cohort over time, which I plotted (Figure 5.14). In these cohorts, SOD1$_{G93A}$/RIPK3$^+/+$ mice as a group appear to have accelerated weight loss compared to the other groups (red arrow). A possible interpretation is that in my case the cohort of male SOD1$_{G93A}$ mice that were RIPK3$^{+/+}$ were representing a worse population due to random sampling and small sample size. In support of this hypothesis, my study was not sufficiently powered to detect differences in sex, as this was not considered in the initial study design. Perhaps with a larger sample size, I would not see inherent differences in onset in the sexes.

Another interpretation is that the RIPK3$^{-/-}$ mice induced the SOD1$_{G93A}$ male mice to behave mechanistically more like the females. For example, there are previous reports implicating elevated estrogen, a female hormone, in reduced systemic inflammation (Au et al., 2016). Similarly, Shivers et al. (2015) found that estrogen reduces inflammation-induced cytokine levels in rat spinal cord at baseline and following injury (Shivers et al., 2015). While it has been noted that inflammation in neurodegenerative models such as ALS can be both neuroprotective and neurotoxic (McCombe and Henderson, 2011; Sargsyan et al., 2005), it is plausible that careful modulation of the inflammatory response to neuronal injury is important to maintain neuronal integrity.

There is growing evidence that necroptosis machinery can activate inflammation independent of cell death (Najjar et al., 2016; Newton, 2015; Newton and Manning, 2016). Given that RIPK3 has known pro-inflammatory roles, independent of cell death, it is possible that deletion of RIPK3 in male mice leads them to have reduced inflammation, mimicking the effect of estrogen on female mice. This could explain why there is no further improvement in SOD1$_{G93A}$/RIPK3$^{-/-}$ female mice, and no improvement in male SOD1$_{G93A}$/RIPK3$^{-/-}$ mice past the
point of their female littermates. Figure 5.15 shows a representative pathway by which the necroptosis machinery can engage inflammation rather than cell death reproduced from Najjar et al. (2016). Najjar et al. (2016) showed that in primary macrophages, the RIPK1 and RIPK3 necrosome drives pro-inflammatory signaling, rather than necroptosis, when TLR4 is activated by LPS and caspase-8 is inhibited. In mice, this signaling response was sustained during acute inflammatory responses to LPS and did not require exogenous manipulation of caspases. RIPK1 and RIPK3 kinases promoted sustained activation of Erk, cFos, and NF-κB, which were required for inflammatory changes.

Figure 5.15. A Model of RIPK1 and RIPK3 Mediated Cell-Death-Independent Inflammation Proposed by Najjar et al. (2016)

Graphical abstract reproduced from Najjar et al. (2016) *Immunity* 45(1); 45-69. Najjar et al. (2016) showed that in primary macrophages, the RIPK1 and RIPK3 necrosome drives pro-inflammatory signaling, rather than necroptosis, when toll-like receptor 4 is activated by lipopolysaccharide (LPS) and caspase-8 is inhibited. In vivo this signaling response was sustained during acute inflammatory responses to LPS and did not require exogenous inhibition of caspases. RIPK1 and RIPK3 kinases promoted sustained activation of Erk, cFos, and NF-κB, which were required for inflammatory changes.

inflammatory responses to LPS and did not require exogenous inhibition of caspases. RIPK1 and RIPK3 kinases promoted sustained activation of Erk, cFos, and NF-κB, which were required for inflammatory changes such as the production of cytokines and chemokines.
Similarly, Ito et al. (2016) found that activation of RIPK1 kinase-dependent inflammatory pathways in microglia was linked to increased cytokine and chemokine production (Ito et al., 2016). These RIPK3-dependent inflammatory pathways in microglia were increased in mice lacking optineurin (OPTN<sup>−/−</sup>), a mouse model they generated to study the role of optineurin in ALS, and which I introduced in Chapter 1.

Given that increased cytokine and chemokine production correlates with reactive gliosis, this could be one contribution of RIPK3 to the overall phenotype of ALS, given that increased reactive gliosis is linked to progression and disease pathology in ALS mice (Burda and Sofroniew, 2014; Frakes et al., 2014; Schiffer et al., 1996). However, Ito et al. (2016) proposed that this increase in reactive gliosis was not, as I would think, directly linked to motor neuron cytotoxicity. Instead, their proposed mechanism was that in OPTN<sup>−/−</sup> mice, increased inflammation in microglia signals oligodendrocyte cell death via TNF-α-induced necroptosis. Oligodendrocyte degeneration via this mechanism in OPTN<sup>−/−</sup> mice led to subsequent myelin defects, causing axonal degeneration (Figure 5.16 graphically represents this mechanism). This axonal damage was rescued by targeting necroptosis via pharmacologic (necrostatin-1-s treatment, a more stable form of Nec-1), genetic RIPK1 kinase inhibition (RIPK1<sup>D138N/D138N</sup> kinase deficient mice). Ito et al. (2016) also generalized this mechanism to the SOD1<sup>G93A</sup> mouse model, and found similar axonal defects that were rescued by targeting necroptosis genetically in RIPK3<sup>−/−</sup> mice or after necrostatin-1-s treatment.

I would posit that if RIPK3-dependent signaling pathways play a role in increased cytokine and chemokine production by microglia, these signals could be cytotoxic to motor neurons as well. Surprisingly, while Ito et al. did observe some shifts in onset of motor symptoms and neuromuscular junction innervation in SOD1<sup>G93A/RIPK3<sup>−/−</sup></sup> mice, correlating with their proposed
mechanism of axonal damage, they did not evaluate motor neuron number or overall survival as I have done in this chapter.

Figure 5.16. Mechanism of Necroptosis in ALS Proposed by Ito et al. (2016)

In the spinal cord, Optn loss leads to an intrinsic sensitivity to RIPK1-dependent activation of microglia, leading to the release of proinflammatory cytokines including TNFα. Oligodendrocytes, sensitized by Optn loss to undergo RIPK1-dependent necroptosis, degenerate following stimulation by TNFα released from microglia. The death of oligodendrocytes leads to the observed formation of less compact myelin, increased denervation from nerve terminals, and axon degeneration.

In their study, Ito et al. (2016) posited that necroptosis leads to increased inflammation in microglia and subsequent oligodendrocyte degeneration. They observe myelin defects associated with these changes in two models of ALS, OPTN<sup>-/-</sup> and SOD1<sup>G93A</sup>. Intrigued by their findings that necroptosis leads to axonal defects in two mouse models of ALS, I compared spinal cord sections
from wild-type, SOD1<sup>G93A</sup> and OPTN<sup>/−</sup> mice (independently generated by the Shneider lab, Columbia University) via electron microscopy. However, while there were minor myelin abnormalities in the white matter of the spinal cord ventral horn, this phenotype occurred equally in wild-type mice rather than segregating with the SOD1<sup>G93A</sup> and OPTN<sup>/−</sup> mice. Additionally, I did not observe abnormal myelin in the ventral roots of the lumbar spinal cord in any genotype.

5.3.2. Lack of Beneficial Effect of RIPK3<sup>/−</sup> in SOD1<sup>G93A</sup> Mice

Taken together, my in vivo study targeting necroptosis via RIPK3 deletion did not show successful rescue of the ALS phenotype in SOD1<sup>G93A</sup> mice, including onset, survival, grip strength, motor neuron number, and axonal retraction from the neuromuscular junction. There are several possible interpretations of these results. The first is that overall, necroptosis has very little to do with the pathology in SOD1<sup>G93A</sup> mice, and that our in vitro studies were not recapitulating the in vivo context. Another possibility is that because RIPK3 deletion is constitutive from birth, the cells could have adapted compensatory mechanisms to circumvent this loss. In this scenario, multiple redundant pathways contribute to cell death in vivo, and that while RIPK3-dependent necroptosis is a dominant mechanism motor neuron cell death, in the absence of RIPK3 cells adapt alternative ways to die such as by RIPK1/Caspase-8 mediated apoptosis (Feltham et al., 2017). In order to begin to explore one aspect of this possibility, I looked to see whether RIPK1, RIPK3, or Caspase-8 levels were increased in tissue from RIPK3<sup>/−</sup> mice using similar methods developed in Chapters 3 and 4. RIPK1 protein and mRNA levels were not increased in the spinal cord of RIPK3<sup>/−</sup> mice compared to wild-type mice at 12 weeks postnatal age (Figure 5.17, a, c, d). Caspase-8 levels were not increased in embryonic spinal cord samples from RIPK3<sup>/−</sup> mice (Figure 5.17, b). In one sample, MLKL mRNA levels were increased 1.5 fold in the spinal cord of RIPK3<sup>/−</sup> mice at 12 weeks
postnatal age; this would need to be repeated to generate a larger sample size to determine whether this increase is biologically significant (Figure 5.17, d). At least I can say from these attempts there is no obvious increase in cell death machinery as a result of RIPK3 deletion in the spinal cord of these mice.

A third possibility is that, while the in vitro models from which this targeting strategy was derived capture the non-cell-autonomous contribution of astrocytes to motor neuron cell death, the in vivo model recapitulates the additional role of mSOD1 in cell-autonomous motor neuron cell death, by mechanisms I discussed in Chapter 1 such as ER stress and mitochondrial dysfunction. Therefore, if necroptosis is solely the result of non-cell-autonomous mechanisms of mSOD1, targeting this pathway may be insufficient to rescue the motor neuron loss in vivo. Given that targeting apoptosis alone does provide some beneficial effects in SOD1\(^{G93A}\) mice, as I discussed in Chapter 1, it is clear that apoptosis does contribute to motor neuron disease. The absence of Bax, which showed complete rescue of motor neurons both in our in vitro models and in vivo (Gould et al., 2006), suggests that Bax may be involved in both non-cell autonomous and cell-autonomous pathways downstream of mutant SOD1. Is there a dependence of motor neuron necroptosis on Bax? Our in vitro data from Chapter 1 would suggest so, given that both Bax and necroptosis machinery are necessary for motor neuron death. However future studies to evaluate the role of necroptosis in motor neuron death in vivo and whether this pathway indeed engages Bax are necessary to confirm this role.
Figure 5.17. No Evidence of Compensatory Cell Death Mechanism in RIPK3−/− Mice

**a.** Protein was extracted from spinal cord tissue (50 µg) from 19-week-old mice and western blot was performed to reveal RIPK1 as described in Chapter 3 and 4. c Shows quantification of the bands. At this time point, there was no difference in levels of RIPK1 protein in wild-type, SOD1G93A or RIPK3−/−/SOD1G93A mice (multiple unpaired t-tests, two-tailed, p>0.05) n=3 biological replicates per condition. **b.** qPCR was performed on extract from the spinal cord of mouse embryos at E12.5 of WT or RIPK3−/− mice using techniques defined in Chapters 3 and 4. There was no change in caspase-8 or RIPK1 mRNA levels between conditions (trend, no statistical test, n=2). **d.** qPCR was performed on extract from the spinal cord of adult WT or RIPK3−/− mice at 12 weeks using techniques defined in Chapters 3 and 4. There was no change in RIPK1 mRNA levels between conditions (trend, no statistical test, n=1). There was a 5-fold decrease in RIPK3 mRNA levels (n=1). There was a 1.5-fold increase in MLKL mRNA (trend, no statistical test, n=1).
Chapter 6: Discussion

The mechanisms of neuronal cell death have been a primary focus of investigation for researchers of neurological disorders and, in particular, neurodegeneration. A complete understanding of these mechanisms could provide crucial insights into the complex biology of these disorders of the nervous system, and could also enable the development of targeted and effective therapies. My work in the Przedborski lab focused on delineating the pathogenic significance of necroptosis in ALS. Given its molecularly-controlled nature, I also sought to identify potential targets for therapeutic purposes. Toward this overarching goal, my work has been divided into four parts which can be defined as follows: (1) determine the role of necroptosis in neuron death in vitro models of ALS (Chapter 2); (2) shed light into the motor neuron-specific regulatory network of necroptosis (Chapter 3); (3) validate key reagents required to study necroptosis and use them in mouse and human postmortem tissue samples to generate histological data about necroptosis engagement (Chapter 4); and (4) test the effects of targeting a central determinant of necroptosis, namely RIPK3, on the diseases phenotype in a mouse model of ALS (Chapter 5).

Although interpretations and discussions of my results took place, for the most part, in each respective chapter, I now provide additional elements of discussion mostly geared toward questions unresolved by my work and about future directions regarding this work.

In Chapter 2, I showed that non-cell autonomous motor neuron cell death resulting from ALS astrocytes involves core components of the necroptosis machinery, namely RIPK1, RIPK3, and MLKL. While our data supporting the involvement of MLKL are compelling, as indicated in
Chapter 2, they rest on the use of a small molecule in our humanized model only. This factor is so critical in our understanding as to how neuronal death occurs, that it would be reassuring to complete this work by using additional pharmacological inhibitors as recently reported by Hildebrand et al. (2014) and/or effective viral vectors to silence MLKL in motor neurons. Strengthening the potential involvement of MLKL here is particularly important since it has been shown to be the actual molecular executioner via forming pores through the plasma membrane (Cai et al., 2014; Chen et al., 2014; Dondelinger et al., 2014; Hildebrand et al., 2014). As shown in the previous chapters, I found little evidence for robust expression of mRNA MLKL in motor neurons and spinal cord tissue, contrasting with the known abundant expression of mRNA Bax. Could it be that Bax in the case of motor neurons and ALS is substituting for MLKL? This idea would be fascinating as Bax-like MLKL is a pore-forming molecule (reviewed by Westphal et al., 2011). Thus, in future studies I would like to examine whether in motor neurons under ALS-related stimuli, Bax rather than translocating to mitochondria translocates to the plasma membrane. Should this happen, I would also like to determine if MLKL is necessary for Bax localization to plasma membrane, for example by heterodimerizing with Bax. Alternatively, I would also wish to perform lipidomic analysis of motor neuron plasma membrane to determine if its lipid composition might have been modified by the diseases process, which, in turn, may promote Bax translocation (Martínez-Abundis et al., 2007).

In Chapter 3, through extracting a gene expression profile of motor neurons undergoing cell death and reverse gene engineering of drivers of the motor neuron cell death phenotype, a number of potential drivers were identified. These drivers will need to be experimentally-validated in future studies for their role in motor neuron death and potential role in necroptosis.
Re-evaluating this data with the goal of uncovering mechanisms of necroptosis, a few observations were made. First, the signature that was obtained was enriched for known drivers necroptosis derived from a screen of mouse fibrosarcoma cells (Hitomi et al., 2008). Second, the master regulators identified driving the death phenotype could be grouped categorically into pathways involving NF-κB, which had been previously validated as a motor neuron death driver, increased TGF-β signaling, and proteins activated during development. The contributions of TGF-β signaling and developmental pathways to motor neuron necroptosis warrant further exploration.

Furthermore, one gene that was increased in the signature, \textit{zfp451}, is a transcription factor that was also identified as a necroptosis driver in mouse fibrosarcoma cells (Hitomi et al., 2008), and was identified as a master regulator of motor neuron cell death (Ikiz et al., 2015). While little is currently known about \textit{zfp451}, its regulon is enriched in functional pathways that could be related to ALS and to necroptosis. Therefore, understanding the role of \textit{zfp451} during motor neuron death and necroptosis could be an interesting area of exploration.

While this data generated interesting leads, a more directed screen for necroptosis could have uncovered additional insight into the regulatory network of necroptosis in motor neurons. For instance, it would have been quite powerful to run a new unbiased reverse gene engineering analysis by exposing motor neurons to ALS astrocyte-conditioned media, as was done in Chapter 2, but this time with or without targeting necroptosis drivers, such as RIPK1, either by pharmacologic or genetic inhibition. With this design, the idea would be to rescue the cell death in a necroptosis-dependent manner and identify those elements of the motor neuron death signature reversed by blocking RIPK1. Thus, from these proposed experiments, a necroptosis-specific signature could be extracted from motor neurons. Once completed, like before, I would propose to validate these candidate necroptosis drivers by silencing each in motor neurons and see if down-
regulating these factors protect against death. With this design in mind, I attempted to carry out such an experiment. My pilot run showed feasibility but the data generated were too noisy to be usable. In addition, and likely as a result, biological replicates also did not show clustering as I would have expected. Thus, this attempt will have to be repeated. At the bioinformatics level, it may be interesting to try to force my future analysis through the Bax regulator network, since we know how significant Bax is here. One challenging aspect of using Bax as an extra-constraint is the fact that Bax is not a master regulator, i.e., transcription factor. One element known to activate the Bax regulatory network is p53 (Miyashita and Reed, 1995). However, p53 was not a master regulator identified by Ikiz et al. (2015) to drive motor neuron death, and inhibiting p53 activity did not rescue motor neuron death, as demonstrated by the work of Diane Re.

In Chapter 4, I showed that while there are increases in RIPK1, RIPK3, and MLKL mRNA in the spinal cords of symptomatic SOD1\textsuperscript{G93A}, the levels of RIPK3 and MLKL mRNA in the spinal cord are quite low, and these proteins are undetectable by western blotting using the given techniques. Do RIPK3 and MLKL indeed play a role in motor neuron death given their levels are quite low? One possibility is that RIPK3 and MLKL are only expressed in a small subset of cells. The most optimal way to study this would be to identify trustworthy immunoreagents against RIPK1 and MLKL to look for localization of these proteins in specific cell types in mouse spinal cord, and their activation at various time points during disease progression in SOD1\textsuperscript{G93A} mice. Perhaps, non-radioactive in situ hybridization could be used to provide at least approximation, but, since most of the cascade of necroptosis is regulated at the post-translational level, the value of such approach would be limited. Of note since, we have not found evidence that any of the anti-RIPK1, -RIPK3, -MLKL immunoreagents are valid for immunohisto- or immunocytochemistry, we are not confident about the data published by several groups over the past few years about the
use of the exact same antibodies to study necroptosis in tissues (Ito et al., 2016; Kanno et al., 2015; Ofengeim et al., 2015). More work would be needed to resolve this important dilemma.

To further answer the question, in Chapter 5, genetic targeting of RIPK3 in the SOD1\textsuperscript{G93A} mouse model was performed. Using a constitutive RIPK3 knockout mouse model (generated by Newton et al., 2004), I generated SOD1\textsuperscript{G93A}/RIPK3\textsuperscript{-/-} mice and evaluated onset, survival, grip strength and motor and axonal pathology during the progression of the disease. While some modest improvements in onset and survival were observed in male mice, these did not correlate with motor function or pathological hallmarks. Taken together, targeting RIPK3 in the SOD1\textsuperscript{G93A} model did not show meaningful improvement of the phenotype. Since the germline deletion of RIPK3 is constitutive, we cannot exclude that some compensatory mechanisms did develop in these mice which, in turn, did mask the true role of this necroptosis factor in the disease phenotype. Should conditional RIPK3 knockout mice become available to us, the study with transgenic SOD1\textsuperscript{G93A} mice might be worth repeating.

Given that RIPK1 has many non-redundant roles in cell death signaling (Dondelinger et al., 2015; Feltham et al., 2017), it would be interesting to see if targeting RIPK1 in motor neurons would have any benefit that we did not capture by RIPK3 deletion. Ito et al. (2016) did explore targeting RIPK1 kinase function in SOD1\textsuperscript{G93A} mice through early administration of necrostatin-1s (a more stable form of necrostatin-1 with increased specificity for RIPK1). While oral administration of necrostatin-1s correlated with a shift in onset of motor symptoms in those mice, the authors did not evaluate overall survival or look at later time points for protection of motor neuron loss, which a future study should explore. In addition, given that we found that in vitro silencing of RIPK1 in motor neurons did not have the deleterious effect as in other cells, as I discussed in the previous chapters, it would be interesting to see whether silencing RIPK1...
specifically in motor neurons would have any benefit that we did not capture by *RIPK3* constitutive deletion. A targeted deletion strategy by in vivo viral delivery of *RIPK1* shRNA to motor neurons, which we have established in the Przedborski lab for other targets, would be an optimal method to evaluate the hypothesis that motor neuron death can occur from multiple redundant RIPK1-dependent signaling pathways. I began working toward this strategy during my thesis work. However, this work in part relies on identification of reliable immunoreagents in order to evaluate whether subsequent RIPK1 protein levels are indeed reduced in motor neurons in the spinal cord following targeted silencing of RIPK1, which I sought to identify in Chapter 3.
Chapter 7: Experimental Procedures

Most methods and reagents used in my work were identical to those previously published by the Przedborski lab and have been cited throughout this document. As such, only new or significantly modified methods are detailed below.

7.1. Mouse Motor Neuron Cultures

Primary spinal motor neuron cultures were prepared as in Nagai et al. (2007) and Re et al. (2014) using embryonic day 12.5 (E12.5) old transgenic embryos expressing GFP driven by the mouse Hb9 promoter (Wichterle et al., 2002). Briefly, cells were plated at 1,500 eGFP+ cells/mL for Hb9::eGFP either on coverslips coated with 0.01% poly-D-lysine and 15 µg ml–1 laminin (poly-D-lysine/laminin) or on astrocyte monolayers. The culture medium was either motor neuron medium supplemented with a cocktail of trophic factors composed of 0.5 ng/ml glial cell-derived neurotrophic factor (GDNF), 1 ng/ml brain-derived neurotrophic factor (BDNF) and 10 ng/ml ciliary neurotrophic factor (CNTF, trophic factor cocktail, R&D Systems); or astrocyte-conditioned motor neuron medium supplemented with trophic factors. Primary MNs cultured from embryos that did not express GFP under the HB9 promoter were quantified using SMI32+ immunostaining (1/500, Sigma #NE1023).

Mouse ES-derived spinal motor neurons, expressing cell surface antigen CD2 and eGFP under the HB9 promoter, were differentiated from ES cells as described in (Wichterle et al., 2002) and purified prior to plating by magnetic activated cell sorting as described in (Ikiz et al., 2015).
7.2. Lentiviral-mediated Gene Silencing in Mouse Primary Neurons

All shRNAs used in this study were constructed in pLKO.1-puro plasmids (Sigma MISSION®). This plasmid contains a puromycin resistance cassette inserted behind a human phosphoglycerate kinase eukaryotic promoter. For mouse Ripk1 silencing, the clone TRCN0000022468 was used. As control, SHC002H, a pLKO.1-puro expressing a non-mammalian targeting sequence was used. Prior to plating, primary motor neurons were transduced with lentiviral particle at multiplicity of infection (MOI) 20 by spinoculation as previously described (Re et al., 2014). Briefly, after isolation, cells were counted and placed into sterile 1.5 mL Eppendorf tubes at 100,000 cells/mL with the viral particles at MOI 20. Cells were spun at 800×g for 30 minutes and gently resuspended in fresh motor neuron growth medium and plated onto postnatal astrocyte monolayers. Plating density was 35,000 infected motor neurons on astrocyte layer for co-culture experiments and 15,000 motor neurons on coated-well for determination of knockdown efficiency.

7.3. Western Blotting

7.3.1. Lysis Buffers

7.3.1.1. RIPA Buffer

RIPA buffer consisted of 150 mM NaCl, 25 mM Tris pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM ETDA, 0.1% SDS, protease inhibitor cocktail and phosphatase inhibitor (Sigma, Complete™, PhosSTOP™).

7.3.1.2. Urea buffer
Urea buffer consisted of 6 M urea, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EGTA, 50 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM benzamidine, 1 mM PMSF, 5 mM N-ethylmaleimide, 1 mM DTT, complete protease inhibitor cocktail and phosphatase inhibitor (Sigma, Complete™, PhosSTOP™).

7.3.2. Mouse Tissue Preparation for Western Blotting

Wild-type or mutant SOD1<sup>G93A</sup> mice on a B6/SJL background (stock #002726, Jackson Laboratory) were perfused with ice-cold PBS (Cat. No #14190136, ThermoFisher) for 4 minutes at 10 ml/minute and spinal cords were carefully dissected out from the spinal column. For all Western blots, the lumbar L1-L5 region was dissected out from the rest of the spinal cord in ice-cold PBS under a microscope. Spinal cords were immediately frozen on dry ice and stored at -80°C in pre-weighed 1.5 mL tubes prior to tissue lysing. On the day of the assay, proteins were extracted at a W/V ratio of 1 mg of tissue per 9 µL of RIPA or 6 M urea buffer containing protease and phosphatase inhibitor cocktail. Tissue was homogenized in RIPA lysis buffer on ice using a Dounce homogenizer and placed on ice for 30 minutes, homogenized twice, and centrifuged at 13,000 rpm for 15 minutes. For the 6 M urea lysis buffer, the exact protocol of Ito et al., (2016) was used. Here, tissue was first homogenized in buffer containing all the ingredients except 6 M urea, then sonicated and incubated for 1 hour at 4°C with rotation. After centrifugation at 15,000 rpm for 20 minutes, the supernatant was removed and the pellet was washed twice in PBS (Cat. No #14190136, ThermoFisher). 6 M urea-containing buffer was added and the pellet was resuspended, sonicated, then re-incubated at 4°C with rotation for 1 hr. Protein concentrations were determined using the Bradford Quick Start™ assay (Bio-Rad). Samples were then diluted to 2 µg/µL protein, using ultrapure water and stock sample buffer at a dilution of 1/5 (v/v): (Tris-HCl
pH 6.8, 10% SDS, 25% Glycerol, 5% β-mercaptoethanol, 0.05% Bromophenol blue). Samples were boiled for 10 minutes at 95°C prior to storage at -80°C, and 1.5 minutes prior to loading.

Then, 50-60 µg of protein preparation was loaded per lane onto a 5-12% Bis-Tris gel (NuPAGE®, Thermofisher) using running buffer prepared by diluting commercially-available MOPS buffer (NuPAGE®, Thermofisher) at a 1/20 dilution (v/v) in ultrapure water. After running, proteins were transferred onto 0.45-µm pore-size nitrocellulose membrane using transfer buffer prepared fresh in Tris/Glycine transfer buffer containing 20% methanol and 0.05% SDS at 300 mA/35 V for 1.5 hours. Membranes were rinsed with ultrapure water prior to blotting.

After blocking [Li-Cor® buffer for fluorescent Western blotting, Rockland, Enhanced chemiluminescence (ECL) preparation: 5% non-fat dry milk in 0.1% Phosphate Buffered Saline Tween-20 (PBST) prepared using commercially-available PBS (Cat. No #14190136, ThermoFisher)], the indicated antibodies (Table 4.1) were incubated in a 1/1,000 dilution of antibody (Li-Cor® buffer for fluorescent Western blotting, Rockland, ECL: 0.1% PBST) overnight at 4°C. The next day, anti-mouse β-actin antibody (Sigma) was added at 1/40,000 and incubated for an additional 1 hour at room temperature. Membranes were washed three times for 10 minutes in 0.1% PBST, incubated in secondary antibody (Li-Cor® buffer 1/20000 IR-700 dye conjugated, ECL: HRP-conjugated 1/3000) for 1 hour at room temperature, then washed three times for 10 minutes in 0.1% PBST prior to imaging. ECL (Bio-Rad) was added for 2 minutes prior to imaging (Li-Cor® Odyssey Imaging system).
7.4. ELISA

For the multiplex human cytokine ELISA, astrocyte media without serum were prepared by conditioning for 7 days with confluent astrocyte layers and prepared as in Nagai et al., (2007). ELISA was performed using 100 µL of undiluted human astrocyte-conditioned medium following the manufacturer’s recommendations (EA-4002; Human Cytokine ELISA Plate Array-Colorimetric, Signosis, Santa Clara, CA). Plate was imaged at 450 nm wavelength using 96-well microplate spectrophotometer (Infinite® 200 PRO, Tecan).

7.5. Generation of the Motor Neuron Cell Death Signature

Purification of mouse ES-MN, application of astrocyte-conditioned media for 3 days, RNA extraction and purification, RNA-seq and Reverse Gene Engineering were performed to generate the motor neuron death signature as described extensively in Ikiz et al., (2015) and in the thesis of Burcin Ikiz (https://academiccommons.columbia.edu/catalog/ac:155922).

Re-analysis of the RNA-seq data was performed as follows. While the mapping and generation of the gene expression signature were similar to the data used in Ikiz et al., (2015), the main improvement in the analysis was the use of multi-sample virtual inference of protein activity by enriched regulon (msVIPER) (Alvarez et al., 2016). This new algorithm is an updated platform from the MARINa, used by Ikiz et al. (2015), to infer protein activity based on the differential expression of transcriptional targets using the mouse whole brain interactome (Gene Expression Omnibus Accession #GSE10415) as the cell context-specific interactome.
7.6. Quantitative Reverse Transcriptase PCR

7.6.1. Mouse Tissue Preparation for RNA Extraction

Tissues from wild-type or mutant SOD1<sup>G93A</sup> mice on the B6/SJL background (stock #002726, Jackson Laboratory) were perfused with cell culture grade ice-cold PBS (Cat. No #14190136, ThermoFisher) for 4 minutes at 10 ml/minute and spinal cords were carefully dissected out. For all western blots, the lumbar L1-L5 region was dissected from the rest of the spinal cord in ice-cold PBS under a microscope. Spinal cords were immediately frozen on dry ice and stored at -80°C in pre-weighed 1.5 mL tubes prior to RNA extraction.

RNA was extracted using TRI Reagent (TRI Reagent®, cat. # T9424, Sigma) following the manufacturer’s protocol. Mouse tissues were placed in TRI Reagent and dissociated using a series of syringe needles until the preparation could pass through a 27-gauge needle three times. After initial RNA extraction, DNAse treatment was performed for 25 minutes at 37°C using rDNAseI (Ambion cat #AM2235). After DNAse treatment, an additional phenol-chloroform extraction of the RNA was performed and the pellet was resuspended in DEPC-treated water (ThermoFisher) and quantified by Nanodrop (Thermo Scientific). Next, 1 µg of RNA per cDNA reaction was used for the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher) following manufacturer’s protocol and primed with both random hexamers and oligo(dT) primers.

A three-step real-time qPCR was carried out with the Realplex 4 Mastercycler PCR System (Eppendorf) using SYBR™ Green dye (ThermoFisher).

The following primers were used for the real-time qPCR:
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Oligo name</th>
<th>Oligo sequence</th>
<th>qPCR product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ripk1</td>
<td>mRipk1-E5-J6F</td>
<td>GGAGCCCTATGAGaATGTCA</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>mRipk1-6R</td>
<td>TCTGCTCATTGCTGCTG</td>
<td></td>
</tr>
<tr>
<td>Ripk3</td>
<td>mRipk3-E7-J8F</td>
<td>ATCCTTCCAGgACTGCGAACAA</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>mRipk3-E8R</td>
<td>GCAGCATCTACCTTTTACCCAG</td>
<td></td>
</tr>
<tr>
<td>Mlkl</td>
<td>Mlkl-E3-J4F</td>
<td>CCTGAAGCAAAtGCTCACTAAAACC</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Mlkl-E4R</td>
<td>CACGGAGGTCCAAGATGTCTT</td>
<td></td>
</tr>
</tbody>
</table>

7.7. Mouse Breeding

RIPK3−/− and RIPK3+/+ females (C57Bl/6N) (Newton et al., 2004), kindly provided by Genentech, were bred with male mutant SOD1G93A mice on the C57Bl/6J background (stock# 004435, Jackson Laboratory). Their progeny was assessed for motor function via grip strength tests, motor neuron number in the lumbar spinal cord, and innervation of the neuromuscular junction at the tibialis anterior muscle.

Genotyping for RIPK3 deletion and the mutant SOD1G93A human transgene was performed using tail DNA and PCR using protocols and primer sequences obtained from Newton et al., (2004) and from Jackson Laboratory (SOD1G93A).

7.8. Mouse Behavioral Tests

The loaded grid test was performed as previously described by Barnéoud et al., (1997). Briefly, the mouse was suspended by the tail and allowed to grip a series of grids with increasing weights
(10, 20, 30, 40 g) and a behavioral score was calculated as follows: score= \[\sum (t_w \times W) / \text{body weight}\] where \(t_w\) corresponds to the maximum time the mouse was able to hold the grid at weight \(W\). Body weight is the weight of the animal at the time of the test. A maximum period of 30 seconds was allowed for each weight. The best of three trials was recorded with at least 15 seconds resting period between each trial of the same weight and at least 30 seconds resting period between each weight. Animals were tested twice a week at roughly the same time of day (afternoon defined as 2 pm – 5 pm) throughout the trial.

Inverted grid or “wire-hang” test was performed by first allowing the mouse to grip a grid and then inverting the grid, thus allowing the mouse to hang. Score was recorded as maximum hold time up to 60 seconds, as the best of three trials with at least 3 minutes resting period between each trial. This test was always performed on a day other than the loaded grid test day. Animals were tested once per week at roughly the same time of day (afternoon defined as 2 pm – 5 pm) throughout the trial.

7.9. Quantification of Innervated Neuromuscular Junctions

7.9.1. Preparation of the Tibialis Anterior Muscle

Mice were perfused with 0.1M PBS for 4 minutes at 10 mL/minute followed by ice-cold 4% paraformaldehyde in 0.1 M PBS for 8 minutes at 10 ml/minute. Tibialis anterior muscle was dissected out and incubated in PBS (Cat. No #14190136, ThermoFisher) overnight at 4°C, then transferred to 30% sucrose in 0.12M phosphate buffer at 4°C for at least 48 hours and subsequently placed in OCT (Tissue-Tek®) in molds on dry ice and stored at -80°C prior to cryosectioning.
Cryosections of tibialis anterior (20 µm) were cut and slide-mounted. Neuromuscular junctions were immunolabeled for presynaptic voltage-gated acetylcholine transporter (anti-rabbit VaChT, 1/2000, Sigma-Aldrich, St. Louis, MI) and post-synaptic nicotinic acetylcholine receptors using fluorescent-conjugated alpha-bungarotoxin (BTX; 1/200 α-bungarotoxin conjugated to Alexafluor-594, Invitrogen, Carlsbad, CA).

Neuromuscular junction innervation was quantified by identifying BTX-positive NMJs (red) and determining the extent of colocalization with VACHT (green). Full innervation is considered greater than a 70% overlap of BTX with VaChT, partial at 30-70% overlap, and denervation at 0-30% overlap. Imaging was performed with a fluorescence microscope at 10× magnification. At least 100 neuromuscular junctions on 20 µm tibialis anterior sections were imaged and counted for each mouse.

7.10. Quantification of Motor Neurons in the Mouse Spinal Cord

Mice were perfused with 0.1 M PBS for 4 minutes at 10 ml/minute, followed by ice-cold 4% PFA for 8 minutes at 10 ml/minute. Spinal cords were removed and post-fixed overnight at 4°C. The L4-L5 segment, identified by its ventral roots, was carefully dissected out with a razor blade under a microscope and incubated at 4°C in 10% sucrose in 0.12 M phosphate buffer for at least 48 hours. The spinal cord was then incubated in 7.5% gelatin/10% sucrose solution at 37°C for 1.5 hours, followed by embedding in a gelatin/sucrose solution in a plastic mold at 4°C until firm for not more than 1 day. Cords in gelatin were then cut into blocks and flash frozen for 45 seconds in 2-methylbutane, dry ice cooled to -60°C and stored at -80°C until sectioning. Next, 20 µm sections were cut on a cryostat and every other section was collected for immunostaining. An entire set of
20-µm sections from L4/L5 was counted and the average number of motor neurons per lateral ventral horn was obtained.

Motor neuron number was quantified as number of cytoplasmic ChAT positive neurons per hemisection in the lateral ventral horn of a 20-µm section of lumbar spinal cord. Every other section for the L4-L5 segment was counted using a fluorescence microscope. A neuron would be counted if the nucleus, as identified by a DAPI counterstain, was present in the optical plan. The anti-ChAT antibody (1/100, goat anti-ChAT, Millipore, Billerica, MA) was incubated overnight at room temperature.
Literature Cited


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