Kv2.1 Channel Clustering in the SOD1-G93A Mouse Model of ALS

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KV2.1 CHANNEL CLUSTERING IN THE
SOD1-G93A MOUSE MODEL OF ALS

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science in
Biomedical Engineering

By

JOSHUA CHRISTOPHER HARRIS
B.S.B.E., Wright State University, 2018

2020
Wright State University
WRIGHT STATE UNIVERSITY
GRADUATE SCHOOL

July 28th, 2020

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Joshua Christopher Harris ENTITLED Kv2.1 Channel Clustering in the SOD1-G93A Mouse Model of ALS BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science in Biomedical Engineering.

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ABSTRACT

Harris, Joshua Christopher. M.S.B.M.E., Department of Biomedical, Industrial Systems, and Human Factors Engineering, Wright State University, 2020. Kv2.1 Channel Clustering in the SOD1-G93A Mouse Model of ALS.

Kv2.1 channels mediate slow-activating K\(^{+}\) rectifier current within the membrane of spinal motoneurons (MNs), and they are known to co-localize with other synapses and ion channels. Although Kv2.1 channels are suggested to regulate MN excitability, little research has gone into investigating its potential contribution to MN-altered excitability in Amyotrophic Lateral Sclerosis (ALS). Using the male SOD1-G93A mouse model of ALS, we examined Kv2.1 cluster area and density in lumbar MNs at four key stages of disease progression. In our experiments, MNs were separated by type via SK3 immunoreactivity in order to isolate and compare the responses of disease-resistant (slow; SK3\(^{+}\)) vs. disease-vulnerable (fast; SK3\(^{-}\)) MNs at postnatal (P) days P10, P30, P90, and end-stage (ES; P120-140). Our results show that in disease-resistant MNs cluster area does not change relative to wild-type until ES when it significantly decreases. In disease-vulnerable MNs cluster area is increased at P90 before also significantly decreasing at ES. Additionally, no changes were found in cluster density throughout disease progression. Electrophysiological recordings using the whole-cord in-vitro spinal cord preparation supported an increase in cluster area at P90 by demonstrating lower net excitability in SOD MNs relative to wild-type, further suggesting pathologically decreased MN activity. These results provide critical, novel information on how disease-resistant vs. disease-vulnerable MNs regulate their excitability throughout ALS.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.  BACKGROUND &amp; SIGNIFICANCE</td>
<td>1</td>
</tr>
<tr>
<td>II. HYPOTHESES</td>
<td>9</td>
</tr>
<tr>
<td>III. MATERIALS &amp; METHODS</td>
<td>11</td>
</tr>
<tr>
<td>IV. RESULTS</td>
<td>18</td>
</tr>
<tr>
<td>V.  DISCUSSION</td>
<td>33</td>
</tr>
<tr>
<td>VI. CONCLUSION</td>
<td>46</td>
</tr>
<tr>
<td>VII. REFERENCES</td>
<td>48</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1: Immunolabeling of MNs and identification of their respective types and immunostaining of Kv2.1 and SK3 channels</td>
<td>20</td>
</tr>
<tr>
<td>Kv2.1 clusters, cholinergic boutons, and SK3 clusters are present and co-localize on the same MN in this example of WT tissue at P90</td>
<td></td>
</tr>
<tr>
<td>Figure 2: Temporal changes of Kv2.1 cluster area in WT and SOD mice</td>
<td>23</td>
</tr>
<tr>
<td>Separating data by MN type reveals common and unique changes in Kv2.1 cluster area throughout disease pathogenesis</td>
<td></td>
</tr>
<tr>
<td>Figure 3: The relationship between Kv2.1 cluster size and MN size</td>
<td>30</td>
</tr>
<tr>
<td>Kv2.1 cluster area increases as somatic largest cross-sectional area (LCA) increases with genotype and MN type</td>
<td></td>
</tr>
<tr>
<td>Figure 4: Kv2.1 density across somatic largest cross-sectional area at ES</td>
<td>32</td>
</tr>
<tr>
<td>Kv2.1 cluster density does not change throughout disease progression or MN type</td>
<td></td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1: List of stains and secondary antibodies used in this thesis</td>
<td>17</td>
</tr>
<tr>
<td>Table 2: Statistical analysis of age and genotype effects</td>
<td>24</td>
</tr>
<tr>
<td>Table 3: Statistical analysis of Kv2.1 cluster area</td>
<td>24</td>
</tr>
<tr>
<td>Table 4: Disease-resistant (SK3⁺) cluster area analysis</td>
<td>26</td>
</tr>
<tr>
<td>Table 5: Disease-vulnerable (SK3⁻) cluster area analysis</td>
<td>27</td>
</tr>
<tr>
<td>Table 6: Cluster area analysis of all MNs</td>
<td>28</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

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I also want to thank my committee members Dr. Mary Fendley and Dr. Matthew Sherwood. Through their guidance and experience, I’ve learned that the principles of engineering can be used to tackle problems in any field of study.

I can’t forget the contributions of Teresa Garrett, Matt Murphy, Lori Goss, Dr. David Ladle and his microscopy facility, my fellow lab mates and peers, and everyone else who worked behind the scenes to make this thesis possible. Your contributions were sincere and meaningful, and I thank you all from the bottom of my heart.

These acknowledgements would not be complete without mentioning my family. Everything that led up to this moment was only possible through the hard work, love, and support of my family. Thank you for my love of learning, my tenacity of spirit, and my continual pursuit of self-improvement.

“It's a dangerous business, Frodo, going out your door. You step onto the road, and if you don't keep your feet, there's no knowing where you might be swept off to.”

— J.R.R. Tolkien, The Lord of the Rings
I. BACKGROUND AND SIGNIFICANCE

*Amyotrophic Lateral Sclerosis*

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease that progressively kills both upper motoneurons of the cerebral cortex and brainstem and lower alpha-motoneurons (MN, alpha-MN) of the spinal cord. These effects lead to muscle weakness, atrophy, loss of motor function, and ultimately death through respiratory failure (Charcot, 1874; Rowland & Shneider, 2001). This fatal disease affects roughly 3 in every 100,000 people, and it has a survival time of 3 to 5 years after diagnosis (Hirtz et al., 2007).

ALS was first described over 150 years ago by French neurologist Jean-Martin Charcot (Charcot, 1874). Despite decades of research investigating this disease, very little has been confidently gleaned about its pathogenesis. Due to the heterogeneity of the disease, an average time to symptom onset is difficult to pin down. Furthermore, because ALS is a diagnosis of exclusion, it takes an average of 8 to 15 months after symptom onset to reach a definitive diagnosis (Ralli, Lambiase, Artico, de Vincentiis, & Greco, 2019).

To date, only two drugs with modest beneficial effects have achieved FDA approval for treatment of ALS. Riluzole, an anti-glutamatergic drug, has been shown to increase survival time by a few months (Bensimon, Lacomblez, & Meiningher, 1994). Edaravone, an anti-oxidant drug that targets free-radicals, has achieved much the same results
Although these drugs have been found to increase survival, there is no change to quality of life.

With what little we know about ALS and its pathogenesis, great efforts are being made to discover more about this disease as well as create treatment options for patients affected by it. Much of this research has led to the creation of animal models. In particular, the superoxide dismutase 1 (SOD1) mutant mouse lines have been well characterized.

**SOD1-G93A mouse model**

SOD1 mouse lines have a mutation in the superoxide dismutase 1 gene, and the 15 current models were created by expressing mutant human SOD1 genes in mice (Gurney et al., 1994). Of these models, the G1H (high-copy line with at least 25 copies; HC) line is the most widely characterized and studied mouse model of ALS available (Turner & Talbot, 2008). SOD1-G93A HC mice (hereafter referred to as SOD) exhibit phenotypic traits that are characteristic of ALS, such as muscle weakness and atrophy, motoneuron degeneration and death, and axonal loss (Fischer et al., 2004). The HC line is studied to maximize ALS-like motoneuron degeneration, and compared to the other SOD1-G93A models its pathogenesis progresses much more quickly and aggressively (Chiu et al., 1995).

As a consequence of these mutations, researchers have seen electrical and morphological abnormalities in SOD motoneurons at pre-natal (Pieri, Carunchio, Curcio, Mercuri, & Zona, 2009) and early post-natal time points (P10; post-natal day 10) (Leroy, Lamotte
d'Incamps, Imhoff-Manuel, & Zytnicki, 2014; Pambo-Pambo, Durand, & Gueritaud, 2009; Quinlan, Schuster, Fu, Siddique, & Heckman, 2011). Around P30, at full adulthood, neurodegeneration begins to occur in the form of functional motor unit and neuromuscular junction loss. This occurs selectively in fast-twitch motor units (Fischer et al., 2004; Hegedus, Putman, Tyreman, & Gordon, 2008; S. Pun, A. F. Santos, S. Saxena, L. Xu, & P. Caroni, 2006). Motoneuron death has not been observed until much later around P90. At this time, slow-twitch motor units also begin to degenerate (Hegedus, Putman, & Gordon, 2007; S. Pun et al., 2006). Progressive degeneration and cell death is expected to occur until animal death (ES; P120-P140 set as humane endpoint). As such, these abnormalities and their effect on motoneuron excitability are expected to start early on and progress in magnitude throughout ALS disease pathogenesis (Delestree et al., 2014; S. Pun et al., 2006).

**Motoneuron Type and Selective Degeneration**

Alpha-MNs can be categorized by the contractile properties of the muscles they innervate. Together, these motor units can be separated into three groups: fast-twitch fatigable (FF), fast-twitch fatigue resistant (FR), and slow-twitch fatigue resistant (S) (Burke, Levine, Tsairis, & Zajac, 1973). In mice, the S-type MN group has been shown through electrophysiology to selectively express SK3, an isoform of SK channels (Deardorff et al., 2013). FF-type MNs have been identified by their expression of matrix metalloproteinase 9 (MMP9), an enzyme involved with extracellular matrix breakdown (Kaplan et al., 2014). Osteopontin (OPN), a protein of the extracellular matrix, has been
found to express FR-type MNs in addition to S-type MNs (Misawa & Morisaki, 2018; Morisaki et al., 2016).

Because markers have been found for both slow and fast-type MNs, these groups can be specifically categorized and investigated for further information on their selective degeneration through ALS disease pathogenesis. Because fast-type MNs degenerate at early stages of disease progression, they are often referred to as disease-vulnerable. Slow-type MNs, which degenerate starting at symptom onset, are referred to as disease-resistant (Fischer et al., 2004).

**Kv2.1 Channels and Motoneuron Excitability**

Kv2.1 channels are membrane bound, voltage-gated potassium channels present throughout the central nervous system (Antonucci, Lim, Vassanelli, & Trimmer, 2001; Aras, Saadi, & Aizenman, 2009; Mohapatra et al., 2009; Muennich & Fyffe, 2004). Regardless of location, these channels have been found to underlie delayed rectifier K\(^+\) currents and to regulate repetitive firing (Fletcher et al., 2017; Guan, Armstrong, & Foehring, 2013; Liu & Bean, 2014; Romer, Deardorff, & Fyffe, 2019). In lumbar spinal motoneurons, Kv2.1 channels form high-density clusters apposed to C-bouton synapses on the soma and proximal dendrites. These channels are also co-localized with small-conductance potassium (SK) channels and type 2 muscarinic (m2) receptors (Muennich & Fyffe, 2004; Romer et al., 2014). As a consequence of their slow channel kinetics and high activation thresholds, Kv2.1 channels in spinal MNs have been found to regulate repetitive firing rather than influence single action potentials (Misonou et al., 2004;
Misonou, Mohapatra, & Trimmer, 2005; Mohapatra et al., 2009; Romer, Deardorff, & Fyffe, 2016; Romer et al., 2019; Romer et al., 2014). This regulation most likely occurs during the interspike interval, where Kv2.1 channel conductance affects Na\(^+\) channel availability (Romer et al., 2019). This process, as part of regulating MN excitability, is vital in order to maintain healthy MNs and normal muscle function during all levels of activity.

In the presence of changing motoneuron excitability, Kv2.1 channels rapidly change their clustered state. During periods of hyperexcitability, Kv2.1 channels rapidly de-cluster and disperse throughout the cell membrane (Antonucci et al., 2001; Misonou, Mohapatra, Menegola, & Trimmer, 2005; Misonou et al., 2004; Romer et al., 2016; Romer et al., 2019). This de-clustering causes reduced thresholds and increased channel kinetics, leading to homeostatically reduced firing rate (Misonou et al., 2004; Misonou, Mohapatra, & Trimmer, 2005; Romer et al., 2016; Surmeier & Foehring, 2004). Interestingly, when the hyperexcitable stimulus is removed, Kv2.1 clusters reform in as soon as two hours (Cerda & Trimmer, 2011; Romer et al., 2016; Romer et al., 2014). What’s more, during periods of hypoexcitability, Kv2.1 clusters increase in size (Romer et al., 2019). Channel kinetics at this activity state have yet to be explored.

The mechanism behind this dynamic clustering has shown to be calcineurin-dependent phosphorylation. In resting motoneurons, Kv2.1 is highly phosphorylated (Misonou et al., 2006). In high-activity states, calcineurin-induced de-phosphorylation precedes Kv2.1 channel de-clustering (Misonou, Mohapatra, Menegola, et al., 2005; Misonou et al.,
2004; Misonou, Mohapatra, & Trimmer, 2005; Romer et al., 2019). In low-activity states, clusters are hyper-phosphorylated (Misonou et al., 2006).

**Possible Roles of Kv2.1 Channels in ALS**

Although Kv2.1 channels have yet to be investigated in ALS, a large body of literature suggests their importance in the dynamic disease pathogenesis. Examination of diseased tissue has revealed mixed effects on motoneuron excitability: both hyper- and hypoexcitability have been reported (Bae, Simon, Menon, Vucic, & Kiernan, 2013; Delestree et al., 2014; Martinez-Silva et al., 2018; Pieri et al., 2009). Given the importance of Kv2.1 channels in normal MN firing, changes to MN behavior could be attributed in part to changes in Kv2.1 channel function (Misonou et al., 2006; Misonou et al., 2004; Romer et al., 2019). Literature has suggested that changes in the voltage gating of Kv2.1 channels alone is enough to affect MN firing (Mohapatra et al., 2009). What is more, the selective degeneration seen between MN types may play a role in Kv2.1 channel function at specific time points throughout the disease (Hegedus et al., 2007; Kuo, Binder, & Heckman, 2020; S. Pun et al., 2006).

Clusters of Kv2.1 channels are known to co-localize with m2 receptors, SK channels, and C-boutons in a highly organized and regulated signaling ensemble. This ensemble is hypothesized to revolve around the regulation of Ca²⁺ conductance, as m2 receptors inhibit Ca²⁺ currents and SK channels are activated by Ca²⁺ (Deardorff et al., 2013; Muennich & Fyffe, 2004; Romer et al., 2014). Changes in intracellular calcium have been suggested to contribute to cellular excitotoxicity, which is a mediator of
neurodegeneration. Increased Ca$^{2+}$ current has been linked to ALS pathology (Engelhardt, Siklos, Komuves, Smith, & Appel, 1995), and overexpression of N-type Ca$^{2+}$ channels has been seen in SOD neurons (Chang & Martin, 2016; Pieri et al., 2013). Given that the Kv2.1-included signaling ensemble is heavily dependent on Ca$^{2+}$, and that clusters of Kv2.1 channels themselves are regulated by Ca$^{2+}$-dependent calcineurin, pathological changes in calcium could directly or indirectly affect Kv2.1 channel behavior (Mohapatra & Trimmer, 2006; Romer et al., 2019). These predictions may be further supported by reports of calcineurin inhibition in the SOD model (Ferri et al., 2004; Kim et al., 2019).

Location itself may have an effect on Kv2.1 channel changes under ALS. During periods of induced high activity, Kv2.1 channels rapidly de-cluster and disperse throughout the soma (Misonou et al., 2006; Misonou, Mohapatra, Menegola, et al., 2005; Romer et al., 2019). This dispersion is suggested to be regulated by efficient trafficking vesicles and signaling proteins (O'Connell, Rolig, Whitesell, & Tamkun, 2006). Interestingly, this change in clustering is also suggested to change channel conductance. Clustered Kv2.1 channels have been found to poorly conduct K$^+$, whereas de-clustered channels conduct K$^+$ the most efficiently (O'Connell, Loftus, & Tamkun, 2010). Others suggest that channel proximity rather than cluster state cause the change in conductance (Fox, Loftus, & Tamkun, 2013).

Under normal activity, Kv2.1 clusters are found on the surface of the soma and proximal dendrites. This specific localization may suggest a function for Kv2.1 channels in
regulating incoming signals from the distal dendrites (Misonou, Mohapatra, & Trimmer, 2005). Given that ALS is suggested to be a retrograde disease where motor units, neurites, and neuromuscular junctions degenerate in a “dying-back” fashion towards the soma (Fischer et al., 2004; Hegedus et al., 2007; Saxena et al., 2013), any changes in incoming signals could affect the normal development and function of Kv2.1 channels (Antonucci et al., 2001).
II. HYPOTHESES

Hypothesis 1: Kv2.1 channel clustering is increased over disease progression in mutant SOD alpha-MNs.

Background
Kv2.1 clusters are known to rapidly change their clustered state under changing activity conditions. Kv2.1 clusters are observed to decrease in size under hyperexcitability and increase in size under hypoexcitability (Misonou et al., 2006; Misonou et al., 2004; Romer et al., 2016; Romer et al., 2019; Romer et al., 2014). Mutant SOD alpha-MNs have also demonstrated abnormal activity under ALS disease pathogenesis: namely, researchers have seen early hyperexcitability and later hypoexcitability starting at full adulthood (Bae et al., 2013; Delestree et al., 2014; Devlin et al., 2015; Kuo et al., 2020; Pieri et al., 2009). Therefore, I hypothesized that Kv2.1 channel clustering would be increased in lumbar SOD alpha-MNs as MN activity state changes over disease progression.

Methods
Immunohistochemistry and confocal microscopy will be used to examine the Kv2.1 channel clustering in lumbar WT and SOD alpha-MNs at four important time points along disease pathogenesis.
**Hypothesis 2**: Kv2.1 channel clustering is selectively increased in F-type mutant SOD alpha-MNs.

**Background**

Because F-type MNs are known to degenerate at early time points under ALS disease pathogenesis, they are suggested to be disease-vulnerable (Hegedus et al., 2007; Hegedus et al., 2008; S. Pun et al., 2006). Furthermore, these MNs have been shown to be selectively targeted in the disease: researchers have seen early soma size changes and early MN activity changes compared to the disease-resistant S-type MNs (Devlin et al., 2015; Dukkipati, Garrett, & Elbasiouny, 2018; Martinez-Silva et al., 2018). Therefore, I hypothesized that Kv2.1 channel clustering will be selectively increased in disease-vulnerable, F-type mutant SOD alpha-MNs.

**Methods**

Immunohistochemistry and confocal microscopy will be used to examine Kv2.1 channel clustering by MN type in lumbar WT and SOD alpha-MNs throughout disease pathogenesis. MN type will be determined with SK3 immunoreactivity (Deardorff et al., 2013).
III. MATERIALS AND METHODS

*Ethical approval*

All animal procedures were performed in accordance with the regulations of the Wright State University Laboratory Animal Care and Use Committee (LACUC) and in accordance with federal laws of the United States of America (animal protocols #1010 and #1046). No human subjects were used in this experiment, and as such compliance was achieved with the above protocols rather than through an IRB. Care was taken to minimize pain and distress of the animals at all times.

*Animal genetic background*

All mice were procured from The Jackson Laboratory or bred from these mice to produce male mice with a B6SJL-TG (stock #002726) genetic background (Tg(SOD1*G93A)1Gur). Briefly, B6/SJL hybrid females (stock #100012) were bred with male hemizygote mice expressing the human SOD1 gene with a glycine-to-alanine mutation at amino acid 93 (SOD1-G93A). Male offspring of this pairing were used for all experiments and compared with their noncarrier littermates (WT). As the disease appears to affect males more than females in human pathology, the present study only used males (Herron & Miles, 2012). Food and water was distributed *ad libitum*. Genotyping was performed by Transnetyx using tail clippings. All mutant hemizygous mice (SOD) expressed a high copy of the mutated gene (>25 copies). SOD mice and their WT
littermates were euthanized at four time points: postnatal day 10 (P10), P30, P90, and end-stage (ES). ES was predefined at full hindlimb paralysis and/or the inability to right itself. This generally occurs at P120–P140.

**Animal terminal procedures**

All animals were anesthetized with a lethal dose of EUTHASOL solution (150 mg kg⁻¹, pentobarbital sodium and phenytoin sodium) and transcardially perfused with a vascular rinse (0.01 M phosphate buffer with 0.8% NaCl, 0.025% KCl, and 0.05% NaHCO3, pH 7–8). This was followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7–8. This procedure is terminal as the animal is exsanguinated and has the spinal cord removed.

**Tissue preparation**

In this study, sections were taken from L4 to L6 of the lower lumbar spinal cord. The spinal cord was quickly removed and post-fixed in 4% paraformaldehyde fixative for about 2 hours or overnight. It was then stored in 15% sucrose at 4°C overnight. The transverse sections themselves were then cut on a cryostat at a thickness of about 50 μm and collected in 0.01 M PBS, pH 7–8.

**Spinal cord immunohistochemistry**

Sections were rinsed with PBS-T (0.01 m PBS containing 0.1% Triton-X, pH 7.3), blocked with normal horse serum (10% in PBS-T), and then incubated free floating in mixtures of primary antibodies overnight at 4°C. All antibodies were diluted with PBS-T.
Nissl immunocytochemistry was performed using a 435/455 blue fluorescent Nissl stain (1:100; N-21479, Neurotrace, Life Technologies) to visualize cell bodies. Kv2.1 labelling was performed using antibodies for voltage-gated potassium channel subfamily B member 1 (Kv2.1; 1:1000 dilution; mouse, NeuroMab, Davis, CA, USA; 75-014).

Sections were co-labeled to detect cholinergic boutons (VACHT; 1:1000 dilution; goat, Abcam; RRID: AB_956453) and calcium-activated potassium channel subfamily N member 3 (SK3; 1:1000 dilution; rabbit, Millipore, Billerica, MA, USA; AB5350).

Immunoreactivity was detected with secondaries from Jackson ImmunoResearch (all were cross absorbed with minimal cross reactivity, PBS-T 0.1%; 1:50 dilution, pH 7.4; Table 1), and incubated at room temperature for around 3 hours. Sections were then mounted and coverslipped with Vectashield mounting medium (Vector Laboratories).

**Confocal microscopy and cluster analysis**

Images were obtained on a Fluoview 1000 (Olympus; RRID: SCR_014215) confocal microscope with a 60× oil-immersion objective in 1 μm steps. Alpha-MNs were differentiated on the basis that they were located in Rexed lamina IX, with somatic diameter > 20μm, and that they received synaptic input from both cholinergic boutons and Kv2.1 clusters (Herron & Miles, 2012; Ishihara, Nagatomo, Fujino, Kondo, & Ohira, 2013; Romer et al., 2016). This is shown through immunoreactivity with both VACHT (VACHT-IR) and Kv2.1 (Kv2.1-IR). For typing α-MNs, SK3 immunoreactivity was employed based off earlier work (Deardorff et al., 2013) to label them as either fast (SK3−) or slow (SK3+). Because fast MNs are more vulnerable in ALS than slow MNs (S. Pun et al., 2006), SK3− cells in this study were considered disease-vulnerable cells whereas
SK3\(^+\) cells were considered disease-resistant cells. Following this, a 2D analysis of MNs was performed similar to our earlier work (Dukkipati et al., 2018). This process, based off of somatic morphology and neurochemistry, allowed for an effective analysis of a large sample of MNs from each age and genotype group. Kv2.1 cluster areas were measured using Fluoview software, where regions of interest (ROI) were drawn around the largest cross-sectional area of clusters on every cell. Density was calculated in part by counting the number of clusters at 3 distinct levels within the z-stack and by measuring perimeter.

**Randomization, blinding, and sampling strategy of collected clusters**

These clusters, in addition to the cells they are within, were chosen at random from collected slice images. Image information was coded throughout the study to keep the data collector blinded to animal age and genotype during immunostaining, confocal imaging, and data analysis. MN information was collected from most cells across all animals to minimize bias. In all, at least two spinal cord sections per animal were analyzed from at least two animals per genotype per time point. Additionally, a large sampling strategy was used following our earlier work to collect between 3 and 7 cluster areas per cell, which maximizes biological variability within cells and animals (Dukkipati, Chihi, Wang, & Elbasiouny, 2017; Dukkipati et al., 2018). Cluster area and cluster density were collected from the same cells, where mean values for each were calculated by cell. Care was taken to collect clusters evenly split among WT and SOD animals and among SK3\(^+\) and SK3\(^-\) cells per time point.
Cluster area and cluster density measurements

Within Fluoview, *en face* cluster areas were collected by drawing ROI around the largest cross-section of each cluster. *En face* denotes clusters that are parallel to the slice within the z-stack. These clusters are identified by their circular shape and by their brief inclusion within the z-stack (visible for 2-3 slices) (Deardorff et al., 2013). Based off our earlier work, a large sample of 3 to 7 clusters were collected per cell to maximize biological variability (Dukkipati et al., 2017). *En face* clusters were collected until adequate statistical power (see below) was achieved.

Cluster density was calculated following our methods in Dukkipati et al. (2017) and in Dukkipati et al. (2018), which are based off a modified technique (Alvarez et al., 2011) to include average cluster count. Specifically, density was calculated as the average number of clusters per 100 µm² over perimeter, where cluster count was determined as the average number of clusters (both *en face* and not) at somatic largest cross-sectional area, 2 µm above, and 2 µm below. 2 µm steps were used to prevent double-counting the same clusters.

Statistical analysis

Statistical analyses were experimental, not descriptive. Random sampling was performed with a random selection of slices for both staining and imaging. Image information, most notably animal ID, age, and genotype, was blinded from the data collector. SPSS and Statistica were used for all statistical analyses. Graphs for all results were created in R using the “ggplot2” package in R Studio. Two-way ANOVA analysis was performed to
investigate the effects of age (P10 vs. P30 vs. P90 vs. ES) and genotype (WT vs. SOD) on Kv2.1 cluster area and density (figs. 2 and 4A). Multiple linear regression was used to compare the coefficients of the interactions between somatic largest cross-sectional area and genotype (figs. 3 and 4B, C). For ANOVA, F values are provided in part to indicate significance of the effects. Fisher’s LSD post hoc tests were used to investigate potential sources of any significant differences. For multiple linear regression, β values and p values are provided to examine significance between coefficients. Statistical power was also calculated via SPSS. Normality was not assumed and was checked within the dataset. Groups that did not meet all assumptions for parametric tests were tested with equivalent non-parametric tests. For our one-tailed hypotheses, significance for all test statistics was set at p < 0.05.
Table 1:
List of stains and secondary antibodies used in this thesis

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<td>Thermo Fisher</td>
<td>Neurons</td>
<td>1µl</td>
<td>Blue Fluorescence</td>
<td>N/A</td>
</tr>
<tr>
<td>Kv2.1</td>
<td>Mouse</td>
<td>NeuroMab</td>
<td>Kv2.1 isoform</td>
<td>1:100</td>
<td>488</td>
<td>715-545-150</td>
</tr>
<tr>
<td>SK3</td>
<td>Rabbit</td>
<td>Millipore</td>
<td>SK3 isoform</td>
<td>1:1000</td>
<td>Cy-3</td>
<td>711-165-152</td>
</tr>
<tr>
<td>VAChT</td>
<td>Goat</td>
<td>Abcam</td>
<td>Cholinergic Marker</td>
<td>1:400</td>
<td>647</td>
<td>715-605-417</td>
</tr>
</tbody>
</table>
IV. RESULTS

_Hypothesis 1:_ Kv2.1 channel clustering is increased over disease progression in mutant SOD alpha-MNs

_Hypothesis 2:_ Kv2.1 channel clustering is selectively increased in F-type mutant SOD alpha-MNs

**Significant Results**

For the first time in an ALS mouse model, mutant SOD MNs have shown selective changes in Kv2.1 channel clustering compared to their WT litter mates. In both disease-resistant (slow; SK3⁺) and disease-vulnerable (fast; SK3⁻) MNs, Kv2.1 clusters have shown to significantly decrease in size at a late disease stage time point (ES). Selectively in disease-vulnerable MNs, cluster area has shown to increase at symptom onset (P90) before decreasing significantly at ES. Kv2.1 cluster density does not show any changes across time or genotype. Further analysis by scatter plot at P90 and ES reveals that Kv2.1 cluster area and density have opposite trends across somatic largest cross-sectional area (cell size). As cell size increases, cluster area increases and cluster density decreases.

_MN identification and labeling, and temporal Kv2.1 immuno staining_

To examine the size and density of Kv2.1 clusters in disease-resistant vs. disease-vulnerable MNs, both Kv2.1-IR (fig. 1A) and SK3-IR (fig. 1B) were used to measure
Kv2.1 clusters and to label MN types at four key stages of disease pathogenesis. Alpha-MNs were identified as large (diameter > 20µm), VACHT-positive cells in lamina IX (fig. 1C). Because slow MNs express SK3-IR and fast MNs do not (Deardorff et al., 2013), we used SK3 immunostaining to label slow MNs (SK3+ cells) versus fast MNs (SK3- cells). Because slow MNs are more resistant in ALS than fast MNs (San Pun, Alexandre Ferrao Santos, Smita Saxena, Lan Xu, & Pico Caroni, 2006), SK3+ cells in this study represent disease-resistant cells whereas SK3- cells represent disease-vulnerable cells. Four time points were examined: P10, P30, P90, and end-stage (ES). At postnatal day 10 (P10), electrical and morphological changes have occurred but neurodegeneration has not started (Leroy et al., 2014; Quinlan et al., 2011). At P30, early adulthood has been reached and selective neurodegeneration has started to affect fast SK3- (disease-vulnerable) MNs (S. Pun et al., 2006). At P90, full adulthood has been reached, symptoms emerge, and neurodegeneration has started to affect slow SK3+ (disease-resistant) MNs (Hegedus et al., 2007). At ES, neurodegeneration has led to full paralysis of the mutant SOD model’s hind limbs and failure to right themselves. This generally occurs between P120 and P140.
**Figure 1: Immunolabeling of MNs and identification of their respective types**

Kv2.1 clusters, cholinergic boutons, and SK3 clusters are present and co-localize on the same MN in this example of WT tissue at P90.

A) Kv2.1 immunoreactivity (IR) (green). The pink arrows denote Kv2.1 clusters. B) SK3-IR (red). The yellow arrow denotes the disease-vulnerable (fast; SK3\(^+\)) cell, and the green arrow denotes the disease-resistant (slow; SK3\(^+\)) cell. C) Cholinergic bouton (Vacht)-IR (white). D) Images overlaid to show co-localization.
Kv2.1 normal development is delayed by disease pathogenesis in SOD mice

To investigate the potential involvement of Kv2.1 channels in ALS pathogenesis, Kv2.1 immunostaining was compared between SOD mice and their age-matched WT littermates at each time point between disease-resistant and disease-vulnerable cells (fig. 2). Using two-way ANOVA, data was analyzed for age, genotype, and between-subject interactions.

In disease-resistant cells (SK3\(^+\)), our analysis revealed that Kv2.1 cluster area has statistically significant age effects (\(F = 10.556, p < 0.001, \text{power} = 99.9\%\); Table 2), but no genotype effects. Fig. 2A shows the normal development of Kv2.1 cluster size over time in the SK3\(^+\) cells of WT mice with cluster area increasing linearly from P10 to P30 and then plateauing throughout the P90 and ES time points (compare blue bars across time points in fig. 2A; \(p\) values in Table 3). In SOD mice, on the other hand, Kv2.1 cluster area was found similar between P10 and P30. Cluster size only starts increasing at P90 (compare red bars across time points in fig. 2A; \(p\) values in Table 3), suggesting delayed development of Kv2.1 cluster size by the disease. The observed decrease in Kv2.1 cluster area at ES in SOD mice is most likely due to the death of these cells at late disease stage.

In disease-vulnerable cells (SK3\(^-\)), our analysis revealed age effect and age*genotype interaction effects (\(p < 0.001, \text{power} > 99\%\); Table 2). Similar developmental trends were seen in the WT mice of SK3\(^-\) cells where Kv2.1 cluster area developed linearly between P10 and P30 and then plateaued after that (compare blue bars across time points in fig.
2B; p values in Table 3). In SOD mice, we again see delayed development that only starts at P90 (compare red bars across time points in fig. 2B; p values in Table 3). These data suggest that disease pathogenesis delays the normal development of Kv2.1 cluster size in both disease-resistant and disease-vulnerable cells.
Figure 2: Temporal changes of Kv2.1 cluster area in WT and SOD mice

Separating data by MN type reveals common and unique changes in Kv2.1 cluster area.

Mean Kv2.1 cluster areas of disease-resistant SK3⁺ (A), disease-vulnerable SK3⁻ (B), and all (C, for both SK3⁺ and SK3⁻) for both SK3⁺ and SK3⁻ cells at four time points comparing WT vs. SOD MNs. The number of cells collected per group is listed within each bar. Error bars indicate standard deviation of the mean. * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001.
### Table 2:
Statistical analysis of age and genotype effects

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Effects</th>
<th>Power</th>
<th>WT cell count</th>
<th>SOD cell count</th>
<th>F value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster Area, All Cells</td>
<td>Age</td>
<td>100.0%</td>
<td>246</td>
<td>241</td>
<td>23.323</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Genotype</td>
<td>70.7%</td>
<td>246</td>
<td>241</td>
<td>6.302</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>Age*Geno</td>
<td>99.9%</td>
<td>246</td>
<td>241</td>
<td>11.081</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cluster Area, SK3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Age</td>
<td>99.9%</td>
<td>120</td>
<td>124</td>
<td>10.556</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Genotype</td>
<td>71.4%</td>
<td>120</td>
<td>124</td>
<td>6.429</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>Age*Geno</td>
<td>67.7%</td>
<td>120</td>
<td>124</td>
<td>2.841</td>
<td>0.039</td>
</tr>
<tr>
<td>Cluster Area, SK3&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Age</td>
<td>100.0%</td>
<td>126</td>
<td>117</td>
<td>12.998</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Genotype</td>
<td>19.3%</td>
<td>126</td>
<td>117</td>
<td>1.193</td>
<td>0.276</td>
</tr>
<tr>
<td></td>
<td>Age*Geno</td>
<td>99.3%</td>
<td>126</td>
<td>117</td>
<td>8.366</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cluster Density, All Cells</td>
<td>Age</td>
<td>100.0%</td>
<td>272</td>
<td>265</td>
<td>22.679</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Genotype</td>
<td>50.8%</td>
<td>272</td>
<td>265</td>
<td>3.938</td>
<td>0.048</td>
</tr>
<tr>
<td></td>
<td>Age*Geno</td>
<td>14.2%</td>
<td>272</td>
<td>265</td>
<td>0.455</td>
<td>0.714</td>
</tr>
<tr>
<td>Cluster Density, SK3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Age</td>
<td>100.0%</td>
<td>142</td>
<td>145</td>
<td>12.477</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Genotype</td>
<td>38.3%</td>
<td>142</td>
<td>145</td>
<td>2.783</td>
<td>0.096</td>
</tr>
<tr>
<td></td>
<td>Age*Geno</td>
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<td>142</td>
<td>145</td>
<td>1.359</td>
<td>0.256</td>
</tr>
<tr>
<td>Cluster Density, SK3&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Age</td>
<td>99.8%</td>
<td>130</td>
<td>120</td>
<td>9.686</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Genotype</td>
<td>23.2%</td>
<td>130</td>
<td>120</td>
<td>1.513</td>
<td>0.220</td>
</tr>
<tr>
<td></td>
<td>Age*Geno</td>
<td>7.7%</td>
<td>130</td>
<td>120</td>
<td>0.147</td>
<td>0.932</td>
</tr>
</tbody>
</table>

### Table 3:
Statistical analysis of Kv2.1 cluster area

<table>
<thead>
<tr>
<th>SK3&lt;sup&gt;+&lt;/sup&gt; Cells</th>
<th>p Values</th>
<th>Power</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P10 vs. P30</td>
<td>P30 vs. P90</td>
</tr>
<tr>
<td>WT</td>
<td>0.018</td>
<td>0.066</td>
</tr>
<tr>
<td>SOD</td>
<td>0.352</td>
<td>0.004</td>
</tr>
<tr>
<td>SK3&lt;sup&gt;-&lt;/sup&gt; Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>0.045</td>
<td>0.185</td>
</tr>
<tr>
<td>SOD</td>
<td>0.483</td>
<td>0.000</td>
</tr>
<tr>
<td>All Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>0.002</td>
<td>0.027</td>
</tr>
<tr>
<td>SOD</td>
<td>0.969</td>
<td>0.000</td>
</tr>
</tbody>
</table>
**Kv2.1 cluster area is exclusively increased in disease-vulnerable cells at symptom onset**

When post-hoc analysis was conducted between WT and SOD cluster area at each time point, our results showed that Kv2.1 cluster area in disease-resistant cells was similar between WT and SOD mice at all time points except for at ES when it decreased significantly due to cell death (compare blue and red bars in fig. 2A; Table 4). However, in disease-vulnerable cells, Kv2.1 cluster area was similar between WT and SOD mice at P10 and P30 time points, increased at P90, and then decreased at ES due to cell death (compare blue and red bars in fig. 2B; Table 5).

When both SK3\(^+\) and SK3\(^-\) MNs were pooled together and their age and genotype effects were analyzed, the data - which have high statistical power due to the large sample size at each time point (Table 2) - confirmed the trends observed above. We observe that motoneuronal Kv2.1 cluster area develops linearly starting at P10 and plateaus after P30 in WT MNs, but in SOD MNs this development is delayed until P90 (fig. 2C; p values in Table 3). At ES, the Kv2.1 cluster area is reduced in SOD MNs. Because the increase in Kv2.1 cluster area at P90 is only driven by SK3\(^-\) cells, the data from all cells had an increasing trend in Kv2.1 cluster area at that time point but it did not reach statistical significance. Interestingly, a new trend appeared at P30 in which SOD Kv2.1 cluster area showed a decrease relative to WT (fig. 2C, p = 0.031; Table 6). This decrease in SOD Kv2.1 cluster area was successfully detected because of the large sample size of cells and because both SK3\(^+\) and SK3\(^-\) cells display this trend albeit modestly (compare the red and blue bars at P90 in figs. 2A and B).
Table 4:

Disease-resistant (SK3+) cluster area analysis

All measured cells were identified from lumbar spinal cord slices from male mice. Mean values ± standard deviations (range of values) and number of cells in each group are noted, along with the p value for statistically significant results from Fisher’s LSD post hoc tests performed after two-way ANOVA analysis.

<table>
<thead>
<tr>
<th>Age</th>
<th>WT MN CA</th>
<th>SOD CA</th>
<th>Statistical Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>P10</td>
<td>8.00 ± 1.65 µm²</td>
<td>8.11 ± 2.21 µm²</td>
<td>Not Significantly Different</td>
</tr>
<tr>
<td></td>
<td>(4.82 - 11.42 µm²)</td>
<td>(4.65 - 12.21 µm²)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 23 from 2 mice</td>
<td>n = 23 from 2 mice</td>
<td></td>
</tr>
<tr>
<td>P30</td>
<td>9.54 ± 2.41 µm²</td>
<td>8.70 ± 1.70 µm²</td>
<td>Not Significantly Different</td>
</tr>
<tr>
<td></td>
<td>(3.73 - 14.21 µm²)</td>
<td>(5.33 - 12.84 µm²)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 31 from 3 mice</td>
<td>n = 33 from 2 mice</td>
<td></td>
</tr>
<tr>
<td>P90</td>
<td>10.61 ± 2.34 µm²</td>
<td>10.4 ± 2.58 µm²</td>
<td>Not Significantly Different</td>
</tr>
<tr>
<td></td>
<td>(5.75 - 16.07 µm²)</td>
<td>(6.31 - 15.90 µm²)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 33 from 3 mice</td>
<td>n = 30 from 3 mice</td>
<td></td>
</tr>
<tr>
<td>ES</td>
<td>10.02 ± 2.84 µm²</td>
<td>7.88 ± 2.44 µm²</td>
<td>p &lt; 0.001 (2-way ANOVA, Fisher's LSD post-hoc)</td>
</tr>
<tr>
<td></td>
<td>(4.99 - 16.06 µm²)</td>
<td>(4.61 - 14.73 µm²)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 33 from 3 mice</td>
<td>n = 38 from 3 mice</td>
<td></td>
</tr>
</tbody>
</table>
Table 5:

Disease-vulnerable (SK3\textsuperscript{\textprime}) cluster area analysis

All measured cells were identified from lumbar spinal cord slices from male mice. Mean values \(\pm\) standard deviations (range of values) and number of cells in each group are noted, along with the p value for statistically significant results from Fisher’s LSD post hoc tests performed after two-way ANOVA analysis.

<table>
<thead>
<tr>
<th>Age</th>
<th>WT MN CA</th>
<th>SOD CA</th>
<th>Statistical Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>P10</td>
<td>8.64 (\pm) 1.78 (\mu\text{m}^2)</td>
<td>9.33 (\pm) 2.07 (\mu\text{m}^2)</td>
<td>Not Significantly Different</td>
</tr>
<tr>
<td></td>
<td>(4.89 - 11.85 (\mu\text{m}^2))</td>
<td>(6.15 - 14.84 (\mu\text{m}^2))</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 27 from 2 mice</td>
<td>n = 28 from 2 mice</td>
<td></td>
</tr>
<tr>
<td>P30</td>
<td>9.85 (\pm) 1.74 (\mu\text{m}^2)</td>
<td>8.91 (\pm) 1.62 (\mu\text{m}^2)</td>
<td>Not Significantly Different</td>
</tr>
<tr>
<td></td>
<td>(7.16 - 13.23 (\mu\text{m}^2))</td>
<td>(6.65 - 13.15 (\mu\text{m}^2))</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 33 from 3 mice</td>
<td>n = 30 from 2 mice</td>
<td></td>
</tr>
<tr>
<td>P90</td>
<td>10.62 (\pm) 2.29 (\mu\text{m}^2)</td>
<td>12.03 (\pm) 2.78 (\mu\text{m}^2)</td>
<td>P = 0.014 (2-way ANOVA, Fisher's LSD post-hoc)</td>
</tr>
<tr>
<td></td>
<td>(7.09 - 16.18 (\mu\text{m}^2))</td>
<td>(8.21 - 20.05 (\mu\text{m}^2))</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 31 from 3 mice</td>
<td>n = 36 from 3 mice</td>
<td></td>
</tr>
<tr>
<td>ES</td>
<td>10.68 (\pm) 2.71 (\mu\text{m}^2)</td>
<td>8.20 (\pm) 3.07 (\mu\text{m}^2)</td>
<td>P &lt; 0.001 (2-way ANOVA, Fisher's LSD post-hoc)</td>
</tr>
<tr>
<td></td>
<td>(6.06 - 16.21 (\mu\text{m}^2))</td>
<td>(3.74 - 14.08 (\mu\text{m}^2))</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 35 from 3 mice</td>
<td>n = 23 from 3 mice</td>
<td></td>
</tr>
</tbody>
</table>
Table 6:  
Disease-resistant (SK3⁺) and disease-vulnerable (SK3⁻) cluster area analysis  

All measured cells were identified from lumbar spinal cord slices from male mice. Mean values ± standard deviations (range of values) and number of cells in each group are noted, along with the p value for statistically significant results from Fisher’s LSD post hoc tests performed after two-way ANOVA analysis.

<table>
<thead>
<tr>
<th>Age</th>
<th>WT MN CA</th>
<th>SOD CA</th>
<th>Statistical Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8.35 ± 1.74 µm²</td>
<td>8.78 ± 2.20 µm²</td>
<td>Not Significantly Different</td>
</tr>
<tr>
<td>P10</td>
<td>(4.82 - 11.85 µm²)</td>
<td>(4.65 - 14.84 µm²)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 50 from 2 mice</td>
<td>n = 51 from 2 mice</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.70 ± 2.08 µm²</td>
<td>8.80 ± 1.65 µm²</td>
<td>P = 0.031 (2-way ANOVA, Fisher's LSD post-hoc)</td>
</tr>
<tr>
<td>P30</td>
<td>(3.73 - 14.21 µm²)</td>
<td>(5.33 - 13.15 µm²)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 64 from 3 mice</td>
<td>n = 63 from 2 mice</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.62 ± 2.3 µm²</td>
<td>11.29 ± 2.79 µm²</td>
<td>Not Significantly Different</td>
</tr>
<tr>
<td>P90</td>
<td>(5.75 - 16.18 µm²)</td>
<td>(6.31 - 20.05 µm²)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 64 from 3 mice</td>
<td>n = 66 from 3 mice</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.36 ± 2.78 µm²</td>
<td>8.00 ± 2.67 µm²</td>
<td>P &lt; 0.001 (2-way ANOVA, Fisher's LSD post-hoc)</td>
</tr>
<tr>
<td>ES</td>
<td>(4.99 - 16.21 µm²)</td>
<td>(3.74 - 14.73 µm²)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 68 from 3 mice</td>
<td>n = 61 from 3 mice</td>
<td></td>
</tr>
</tbody>
</table>
Kv2.1 cluster size increases as MN size increases

To investigate whether Kv2.1 cluster area scales with MN size, and whether this projected scaling might be affected throughout disease pathogenesis in disease-resistant versus disease-vulnerable cells, we compared Kv2.1 cluster area against somatic largest cross-sectional area (LCA) by MN type and by genotype (fig. 3). Because the Kv2.1 cluster area at P10, P30 and P90 have similar cell distributions in the WT and SOD MNs, we focused our presentation here on P90 as a representative time point (fig. 3A, C) and on ES (fig. 3B, D) as an illustration of what happens at late disease stage when significant degeneration has taken place.

Generally, Kv2.1 cluster area was found to increase linearly with cell size in WT MNs, indicating a linear proportional relationship between Kv2.1 cluster size and cell size. This trend was seen in both SK3⁺ and SK3⁻ cells at P90 and at ES (fig. 3, blue lines). In SOD MNs, this linear proportional scaling of Kv2.1 cluster area with MN size was generally maintained throughout disease in disease-resistant (SK3⁺) cells, although the slope of this relationship was slightly increased and shifted downwards at ES (not statistically significant, fig. 3A, B, red lines). Disease-vulnerable (SK3⁻) cells still showed linear proportional scaling of Kv2.1 cluster area with MN size. This scaling was initially shifted upwards across all MN sizes at P90 (fig. 3C, red line), reflecting the increase in Kv2.1 cluster area described in fig. 2B. However, at ES, the slope of this relationship is significantly increased and shifted downwards (fig. 3D, red line). The distribution of SOD SK3⁻ cells relative to WT at ES was statistically significant (Genotype: β = -1.496, p = 0.002; LCA: β = 1.174, p < 0.001).
**Figure 3:** The relationship between Kv2.1 cluster size and MN size

Kv2.1 cluster area increases as somatic largest cross-sectional area (LCA) increases with genotype and MN type.

A) Disease-resistant cells at P90. WT n = 33, SOD n = 30. B) Disease-resistant cells at ES. WT n = 33, SOD n = 38. C) Disease-vulnerable cells at P90. WT n = 31, SOD n = 36. D) Disease-vulnerable cells at ES. WT n = 35, SOD n = 23. * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001.
**Kv2.1 density decreases as MN size increases**

To examine the effects of disease progression on Kv2.1 channels, we also measured Kv2.1 cluster density along with cluster area in all above experiments in disease-resistant and disease-vulnerable cells at P10, P30, P90, and ES. Two-way ANOVA analysis of Kv2.1 cluster density across the four time points showed that Kv2.1 cluster density of SK3+ and SK3− cells was similar between WT and SOD at all four time points (i.e., no genotype effects in both MN types; fig. 4A is an example at ES). Furthermore, the developmental profile of Kv2.1 cluster density was flat over time in WT cells and in SOD cells (i.e., no age effects, data not shown). However, when the relationship between Kv2.1 cluster density and MN size was investigated in SK3+ and SK3− cells, we found a reversed relationship to that of Kv2.1 cluster area with MN size. Specifically, Kv2.1 cluster density was found to decrease linearly with cell size in WT MNs, indicating a linear inversely-proportional relationship between Kv2.1 cluster density and cell size (fig. 4B, C, blue lines). This trend was seen in both WT SK3+ and SK3− cells (fig. 4B, C, blue lines) and was maintained in the disease in disease-resistant (fig. 4B, red line) and disease-vulnerable (fig. 4C, red line) cells.
Figure 4: Kv2.1 cluster density across somatic largest cross-sectional area at ES

Kv2.1 cluster density does not change throughout disease progression or MN type.

A) Mean Kv2.1 density for disease-resistant (SK3⁺), disease-vulnerable (SK3⁻), and all cells at ES in WT vs. SOD MNs. The number of cells collected per group within each bar.

B) Disease-resistant cells at ES. WT n = 29, SOD n = 38.

C) Disease-vulnerable cells at ES. WT n = 34, SOD n = 24. Error bars indicate standard deviation of the mean.
V. DISCUSSION

Kv2.1 clusters represent collections of voltage-gated, delayed-rectifier potassium channels. These channels have generally been suggested to aid in homeostatic suppression by regulating neuronal activity (Misonou, Mohapatra, & Trimmer, 2005; Romer et al., 2016; Romer et al., 2019). Furthermore, literature has shown that Kv2.1 channels dynamically cluster given different activity states (Antonucci et al., 2001; Romer et al., 2016; Romer et al., 2019). Therefore, any changes to their morphology through disease would have significant effects on how they regulate the excitability of MNs. Using the SOD1-G93A mouse model of ALS, Kv2.1 cluster area and density was investigated across four important time points in disease pathogenesis. Differences between disease-resistant (slow; SK3⁺) and disease-vulnerable (fast; SK3⁻) MNs was evaluated. Care was also taken to use a large sample size to ensure that each time point had high statistical power.

Here for the first time, our results implicate Kv2.1 channels in the disease pathogenesis of ALS. Within the SOD1-G93A mouse model, Kv2.1 cluster area was shown to increase at symptom onset (P90) and exclusively in disease-vulnerable cells (fast; SK3⁻). This increase in cluster size suggests low alpha-MN excitability under the ALS disease condition (Romer et al., 2019). Furthermore, because these changes are only seen in a MN type known to degenerate earlier in disease pathogenesis, this may indicate that the
results presented are due to direct disease effects of changing morphology and excitability (Dukkipati et al., 2018; Martinez-Silva et al., 2018; S. Pun et al., 2006; Saxena et al., 2013), rather than compensatory effects. These results provide insights into how Kv2.1 channels are distributed between modes of disease vulnerability, and they also add to the collective story of Kv2.1 channel dynamics. Furthermore, although this story applies to spinal alpha-MNs, our research is relevant to the Kv2.1 channels present throughout the central nervous system (Guan et al., 2013; Liu & Bean, 2014; Mohapatra et al., 2009; Romer et al., 2019).

**SK3 Expression May Offer Greater Insights Into ALS Disease Progression**

Recent studies have shown the importance of studying morphological changes under disease pathogenesis (Dukkipati et al., 2018). In particular, work with fast-type cell markers (MMP9 and osteopontin) has demonstrated the benefits of differentiating between cell type in neurodegenerative disease studies (Kaplan et al., 2014; Morisaki et al., 2016; Yamamoto, Murayama, Takao, Isa, & Higo, 2017). Furthermore, it is well established that fast-type MNs are vulnerable to disease (Hegedus et al., 2008; S. Pun et al., 2006). By including SK3-IR in our study, more insights can be made on the MN types involved in ALS and their potential changes throughout time (Deardorff et al., 2013; Kaplan et al., 2014; Martinez-Silva et al., 2018). Research has also shown that differences between cell types via axonal pruning and excitability start to appear as early as P7 in the SOD model (S. Pun et al., 2006; Saxena et al., 2013).
Through this study, it is shown that the inclusion of SK3-IR can not only reveal specific changes in MN type but also demonstrate how trends in the data can be hidden when all MN types are combined (see fig. 2). Our results show a significant increase in Kv2.1 cluster area at the P90 time point which is only evident when investigating disease-vulnerable (SK3\(^1\)) alpha-MNs. In conclusion, using SK3-IR can further our understanding of how disease-vulnerable and disease-resistant MNs selectively change throughout ALS disease pathogenesis. Selective targeting of these cells at different time points could lead to new avenues for neuroprotection and therapy.

**Increased Kv2.1 Cluster Area May Suggest Pathologically Reduced MN Activity**

Our results include a number of significant changes in Kv2.1 cluster area under ALS disease pathogenesis. In addition to the large-scale decrease in SOD cluster area at ES, this study has shown a selective increase in SOD cluster area at P90 in the disease-vulnerable cells compared to WT. Given the dynamic morphology of this channel, our results suggest likewise dynamic alpha-MN activity states under the disease. Taken together, these results only support our first hypothesis at symptom onset (P90): earlier in the disease (P10 and P30) Kv2.1 channel clustering did not change, and at ES we observed decreases in Kv2.1 cluster area. Because the increase in Kv2.1 cluster area at P90 is only observed in disease-vulnerable, F-type MNs, these results nonetheless support our second hypothesis.

Kv2.1 channels throughout the central nervous system have been found to rapidly de-cluster under increased or prolonged MN activity (Misonou et al., 2004; Misonou,
Mohapatra, & Trimmer, 2005; Romer et al., 2016; Romer et al., 2019). Due to their slow channel kinetics and influence on delayed rectifier current, these channels only play a role during repetitive firing of the cell (Misonou, Mohapatra, Menegola, et al., 2005; Mohapatra et al., 2009). Specifically in alpha-MNs, Kv2.1 channels have been found to regulate interspike interval and rapidly de-cluster when there are prolonged changes in intracellular Ca\(^{2+}\) (Romer et al., 2019). This de-clustering, or decrease in cluster area, is also suggested to be a protective mechanism of surviving alpha-MNs against proposed excitotoxicity at late stage time points. Interestingly, Romer et al. (2019) also saw increases in Kv2.1 cluster area under low-activity conditions, further highlighting the dynamic morphology of this channel under different excitability states. Given that hyperexcitability is a well-known theory in ALS (Bae et al., 2013; Pieri et al., 2013; Pieri et al., 2009), it is critical to consider changes in Kv2.1 cluster size as a reflection of changing cell activity.

In a topical ALS study (Herron & Miles, 2012), Kv2.1 cluster size was investigated at four time points including ES. Interestingly, no differences were reported in genotype across time, suggesting no change in MN excitability. In comparison, our study included approximately twice as many alpha-MNs as the Herron and Miles (2012) study. Furthermore, our study measured changes in cluster area (sq. microns) while Herron and Miles (2012) used Feret’s diameter (cluster length, in microns). While both techniques examine changes in size, differences in reported parameters and sample size make it difficult to synthesize results (Dukkipati et al., 2017). Furthermore, it is important to note
that even small differences in time point within animal models can produce significantly different findings (McCutcheon & Marinelli, 2009).

In other mouse models of neurodegeneration, similar trends were seen in late stage time points. In a mouse model of Spinal Muscular Atrophy (SMA), significant decreases in Kv2.1 channel expression via western blot were found at a late stage in the disease (Fletcher et al., 2017). Additionally, significant decreases in Kv2.1 channel intensity were reported at a late disease stage in a mouse model of Huntington’s Disease (Ariano et al., 2005). Although expression at symptom onset was not investigated, these results are similar to what we found at the ES time point where late stage disease effects occur. Here, MN degeneration leads to cluster dysfunction and break down.

Given the reported trends between alpha-MN activity and Kv2.1 cluster size, our results suggest that Kv2.1 cluster area increases under ALS disease pathogenesis as a consequence of low cellular activity (Romer et al., 2016; Romer et al., 2019). Parallel electrophysiology experiments investigating disease-vulnerable alpha-MNs in mutant SOD1 mice have also reported lower net excitability, further supporting the idea of low cellular activity (Elbasiouny laboratory, unpublished data). This increase in cluster area comes at a late time point where motoneuron degeneration, muscle denervation, and loss of muscle force have been well documented, particularly in large, more vulnerable cells (Hegedus et al., 2007). Hypoexcitability has also been specifically reported in ALS disease pathogenesis (Delestree et al., 2014; Devlin et al., 2015; Martinez-Silva et al., 2018). What is more, some research claims that the reduced excitability seen in disease-
vulnerable MNs actually increases SOD1 protein misfolding, leading to decreased MN survival and function (Saxena et al., 2013).

**Calcineurin Dysfunction is Implicated in ALS Disease Pathogenesis**

In ALS cases, a widespread hallmark of the neuropathology is cellular trans-activating response region DNA binding protein (TDP-43) occlusions. These TDP-43 occlusions are present in 90% of all cases, and they have recently been implicated as a pathological factor of ALS (Chong & Forman-Kay, 2016; Neumann et al., 2006). In both familial and sporadic forms of ALS, these TDP-43 occlusions are found to be hyper-phosphorylated (Ferri et al., 2004; Kim et al., 2019). What is more, this phosphorylation has been found to be regulated by calcineurin, the same phosphatase responsible for Kv2.1 channel clustering (Liachko et al., 2016; Misonou et al., 2004). Research has shown that calcineurin is impaired in ALS, most likely by the overexpressed SOD1 mutations. Because calcineurin is prevented from regulating the phosphorylation of TDP-43 proteins, occlusions form and add to the pathogenesis of ALS (Ferri et al., 2004; Kim et al., 2019). Given that calcineurin directly affects Kv2.1 channel clustering, and that hyper-phosphorylation has been observed in these clusters under low activity states, these findings may suggest that our results are due to calcineurin inhibition as a direct effect of ALS.

**Ionic Changes at the Synapse May Confound Reported Disease Effects**

Within alpha-MNs, these dynamic channels co-localize with multiple other K+ channels as well as cholinergic boutons of the presynaptic terminal and SK channels and type 2
muscarinic (m2) receptors of the postsynaptic terminal (Deardorff et al., 2013; Muennich & Fyffe, 2004). When considering observed changes in Kv2.1 channels, it is also important then to consider potential changes in other co-localized channels on the synaptic signaling ensemble.

Research into cholinergic boutons has produced conflicting size changes under a neurodegenerative disease condition, including increases (Herron & Miles, 2012; Pullen & Athanasiou, 2009; Saxena et al., 2013), decreases (Milan et al., 2015), and no change (Dukkipati et al., 2017; Herron & Miles, 2012; Milan et al., 2015; Pullen & Athanasiou, 2009) in size. With all these reported changes, it is difficult to conclude exactly how they affect MN excitability under disease. However, under normal conditions, cholinergic boutons have been found to regulate MN excitability via the cholinergic activation of m2 receptors (Landoni, Myles, Wells, Mayer, & Akay, 2019; Miles, Hartley, Todd, & Brownstone, 2007). These m2 receptors in turn inhibit SK channel activity, resulting in reduced medium after-hyperpolarization (mAHP) and increased firing frequency (Deardorff et al., 2013; Miles et al., 2007).

Little research has been conducted into SK channels under a disease condition, but literature suggests they regulate burst firing in the spinal cord (Mahrous & Elbasiouny, 2017). Recent immunohistochemical work in our lab has also implied that SK channel cluster size and density decreases under the ALS disease condition (Elbasiouny laboratory, unpublished data). Given that SK channels mediate mAHP (Bond et al., 2004;
Sah & Faber, 2002), this decrease in size may suggest increased MN activity via modified firing frequency.

Interestingly, research into acetylcholine-mediated m2 receptors in the SOD mouse model produce results that are just as mixed as their pre-synaptic activators. Researchers have seen both decreases (Milan et al., 2015) and no change (Herron & Miles, 2012) in cluster size, further adding to the confounding results from cholinergic bouton size investigations. Under normal conditions, m2 receptors are known to regulate MN activity via SK channel inhibition as well as Ca\(^{2+}\) current inhibition (Allen & Brown, 1993; Deardorff et al., 2013; Deardorff, Romer, Sonner, & Fyffe, 2014; Romer et al., 2019).

Taken together, these findings demonstrate the variety of changes occurring under disease pathogenesis as well as normal function. These cellular structures, both pre- and post-synaptic, represent a highly specific and integrated system for precise cell firing. These structures work together to maintain the normal activity of dynamic motoneurons, and this brief summary of their roles demonstrates the importance of further investigating them in context with one another. As it refers to Kv2.1 channels, it is hypothesized that they act as safeguards on the cholinergic bouton (Romer et al., 2019). Under normal activity, SK channels and m2 receptors work in tandem to maintain firing frequency by regulating mAHP and Ca\(^{2+}\) currents (Allen & Brown, 1993; Deardorff et al., 2013). When MN activity is abnormally elevated, and there is an influx of positive ions including Ca\(^{2+}\), calcineurin-dependent de-phosphorylation occurs to rapidly de-cluster Kv2.1 channels and homeostatically reduce firing (Deardorff et al., 2014; Romer et al.,
Given that our reported increase in Kv2.1 cluster area suggests low MN activity, these findings may also allude to dysfunction in other co-localized channels under ALS.

*Kv2.1 Cluster Location May Play a Role in Observed Changes Under ALS*

Kv2.1 clusters are known to reside on the surface of the soma and proximal dendrites, where they are suggested to play a role in mediating the incoming signals from distal dendrites (Misonou, Mohapatra, & Trimmer, 2005). Under ALS, these distal dendrites and their neuromuscular junctions are known to degenerate in a retrograde manner (Fischer et al., 2004; Hegedus et al., 2007). What is more, motor unit loss and degeneration has been seen in large, disease-vulnerable MNs as soon as P40 (Hegedus et al., 2007). Given that fast-type MNs are suggested to be disease-vulnerable because of their low cellular excitability (Leroy et al., 2014; Martinez-Silva et al., 2018; S. Pun et al., 2006; Saxena et al., 2013), and because they have shown to be selectively enlarged in the disease (Dukkipati et al., 2018), our results at P90 may further suggest pathologically reduced MN activity. What is more, given that Kv2.1 cluster area was only increased in large, disease-vulnerable MNs, these findings further support the idea of direct disease effects.

*Kv2.1 Cluster Density Stays Constant Except Over Somatic Largest Cross-Sectional Area*

Cluster density, as used in this thesis, is an average of cluster count per 100µm² over perimeter. Within its calculation, the number of clusters at three unique locations and the size of the respective MN itself is considered (see *Materials and Methods: Cluster*
Throughout this investigation, Kv2.1 cluster density did not change over time, genotype, or MN type. Even at ES, where large-scale changes are known to occur due to the disease, SOD cluster density is not different from WT. These findings are interesting in comparison to topical literature: under increased neuronal activity, cluster count and other analogues for cluster density have shown to decrease (Cerda & Trimmer, 2011; Misonou et al., 2004; Mulholland et al., 2008). Given that our results do not imply increased MN activity via calcineurin-mediated de-phosphorylation and de-clustering (Misonou et al., 2006), and that calcineurin itself is inhibited by ALS (Ferri et al., 2004; Kim et al., 2019), our results suggest that Kv2.1 cluster density does not change in the face of reported altered MN firing under disease pathogenesis.

Careful statistical examination of Kv2.1 cluster density reveals no age effects (data not shown) or genotype effects (fig. 4A). Furthermore, analysis of average cluster count over time reveals no genotype effects (data not shown). It is interesting then that changes do appear in cluster density trends when expressed over MN size. Data shows that Kv2.1 cluster density decreases as somatic LCA increases in both examined MN types (fig. 4B, C). Considering then that no other parameter of cluster density shows changes, and that WT and SOD cluster density trends themselves show no difference over somatic LCA, this suggests that the trends seen are a computational side effect of increasing MN size and perimeter rather than a direct effect of changing Kv2.1 cluster density. In essence, because of the way it is calculated, cluster density decreases as MN perimeter increases. Because average cluster count itself does not change, the trends seen in Kv2.1 cluster density are a direct effect of MN size.
Kv2.1 Cluster Area and Density Demonstrate Different Relationships to Somatic LCA

Many of the MN experiments conducted today are based off the work of Henneman and his peers. In his seminal 1965 papers, Henneman proposed the “size principle” to describe motor unit order recruitment, firing threshold, and force generation based on MN size. In particular, it is suggested that smaller MNs have lower firing thresholds than larger MNs (Henneman, Somjen, & Carpenter, 1965b). This hypothesis would imply that smaller MNs are able to generate greater synaptic potentials than larger MNs under the same excitatory input. What is more, Henneman suggests that smaller MNs have larger input resistance due to their geometry and size (Henneman & Olson, 1965; Henneman, Somjen, & Carpenter, 1965a; Henneman et al., 1965b).

Taken together, these findings may suggest varying intrinsic electrophysiological properties between MNs of different sizes. In consideration of suggested input resistance changes, these properties may also be affected by changing capacitance via cholinergic input. Because Kv2.1 channels co-localize with cholinergic boutons, Henneman and his work may indicate that the number or size of Kv2.1 clusters per cell would increase as somatic LCA increases. Interestingly, our results show that Kv2.1 cluster area increases as somatic LCA increases (fig. 3) while the cluster density decreases (fig. 4). What is more, Kv2.1 cluster count did not change across time. Taken together, these findings suggest that Kv2.1 clusters exclusively increase in size as somatic LCA increases. Although not directly supporting Henneman, these results would still suggest increased
synaptic surface area and would tentatively support the idea of implied lower input resistance in larger alpha-MNs (Henneman et al., 1965a).

In closing, the positive correlation seen between Kv2.1 cluster area and somatic LCA is a natural morphological phenomenon driven by the changing electrophysiological demands of a developing cell. These findings, based off of the “size principle,” suggest that cluster area increases to regulate MN excitability and to maintain motor unit size recruitment. The negative correlation with Kv2.1 cluster density is suggested to be a side effect of increasing MN size rather than a direct change in cluster count.

In light of this, it would be interesting then to revisit the cluster area and cell size distributions at P90 and ES (fig. 3). Besides the slight linear proportional relationship seen across all figure subsets, two main trends can be seen. First of all, it appears that R² values for WT MNs are overall lower across P90 and ES in the disease-vulnerable cells compared to disease-resistant cells (compare blue lines from fig. 3A, B to C, D). These distributions may suggest that the large, fast-type MNs may have more biological variability than the slow-type MNs. The naturally greater range of cell sizes seen with these fast-type MNs may also play a role in our observations. Secondly, in SOD MNs, it appears that R² values and slope increases from P90 to ES in both disease-resistant and disease-vulnerable cells (compare red lines in fig. 3A to B and fig. 3C to D). These changes are most drastic at ES, where a clear cut-off in cell size appears due to large-scale degeneration and cell death (fig. 3B, D). Taken together, these distributions may indicate that the surviving MNs have become smaller and less variable. In addition, the
increased slope of the surviving MNs demonstrates that Kv2.1 cluster area increases faster over cell size, potentially suggesting compensation effects geared towards the preservation of motoneuronal function (Dukkipati et al., 2018).
VI. CONCLUSION

In conclusion, the results from this thesis only support our first hypothesis at certain disease stages while wholly supporting our second hypothesis. SOD Kv2.1 cluster area was selectively increased compared to WT in disease-vulnerable cells just as motor function is deteriorating (P90). Later in disease progression (ES), in both disease-resistant and disease-vulnerable cells, SOD Kv2.1 cluster area was decreased compared to WT. No change was observed between WT and SOD cluster density. This increase in Kv2.1 cluster area suggests low alpha-MN activity under ALS (Misonou et al., 2006; Romer et al., 2019). Evidence also suggests that this is a direct disease effect of the changing alpha-MN activity seen in both mouse and human models of ALS (Dukkipati et al., 2018; Hegedus et al., 2007; Martinez-Silva et al., 2018; S. Pun et al., 2006; Saxena et al., 2013). Although not wholly supporting our first hypothesis, these results provide new and exciting insights into Kv2.1 channel clustering changes under ALS. It is expected that these novel findings, paired with consistent experimental designs, will help to reveal more of the morphological changes underlying selective dysfunction between MN types in the disease progression of ALS.

In addition, cluster area and density reveal opposite trends as cell size increases: cluster area increases and density decreases. Cluster area most likely increases in response to the changing electrophysiological demands of a larger MN. Density most likely decreases as
a side effect of increasing MN size and perimeter rather than a direct consequence of changing cluster count. Further research into Kv2.1 channels and their co-localization with other post-synaptic proteins will serve to better our understanding of how these channels change together throughout the disease process. What is more, investigation of cluster parameter across somatic parameters can help to reveal trends in normal cell morphology. These findings can add to our collective knowledge of alpha-MN behavior and excitability as it relates to changing soma size.
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