SK CHANNEL CLUSTERING IN SOD1-G93A MOTONEURONS

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

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Saihari S Dukkipati ENTITLED SK Channel Clustering in SOD1-G93A Motoneurons BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Dukkipati, Saihari S., M.S. Physiology and Neuroscience, Department of Neuroscience, Cell Biology, and Physiology, Wright State University, 2016. SK Channel Clustering in SOD1-G93A Motoneurons.

Amyotrophic lateral sclerosis (ALS) is a devastating neuromuscular disease that currently has no cure and extremely limited treatment options. The specific mechanisms that underlie motoneuron degeneration and death, which are classical features of this disease, are mostly unknown. This thesis tests the hypothesis that small-conductance calcium-activated potassium channels (SK) may be downregulated in ALS motoneurons, as suggested by computational modelling. SK channel expression was measured in spinal alpha-motoneuron cell bodies or somata of wildtype (WT) and mutant (mt) SOD1-G93A mice, a transgenic animal model of ALS. Quantitative immunohistochemical analysis of the developmental expression of SK channel isoforms SK2 and SK3 at various postnatal time points was performed to assess the effects of motoneuron degeneration on the level and/or pattern of protein expression on the somata of lower lumbar motor nuclei. Results indicate that the selective expression of SK3 may be gradually reduced over development in WT and mutant SOD1 mice but is affected by disease pathogenesis. In addition, SK channels appear to be clustered in both WT and mutant SOD1 motoneurons throughout development. However, SK clusters appear to be significantly smaller in mutant SOD1 motoneurons compared to their WT littermates. These changes indicate that the activity of SK channels, which regulate the firing rate of motoneurons, may be affected in ALS.
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LIST OF ABBREVIATIONS USED

-IR  Immunoreactivity

MN  Motoneuron
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I am indebted to quite a few people for their role in the scientific process and production of this work, as well as the foundations of my graduate and post-graduate career.

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Lastly, but most importantly, I would like to thank my family, who have been nothing but supportive of me at my every fall and rise. I hope to keep you proud.
For my sister...

Now and Always...
I. BACKGROUND & SIGNIFICANCE
ALS

Amyotrophic lateral sclerosis (ALS) is a progressive and non-cell autonomous neurodegenerative disease. Current estimates of prevalence predict a worldwide incidence of 2-4 per 100,000 people and an average survival time of 3–5 years after initial diagnosis (Hirtz et al., 2007). ALS is characterized by selective degeneration and death of upper motoneurons in the cortex and lower alpha-motoneurons (henceforth referred to in this thesis as motoneurons or alpha-MNs) in the brainstem and spinal cord, which leads to muscle weakness, atrophy, paralysis and death (Rowland & Shneider, 2001).

ALS has been studied for almost 150 years since Jean-Martin Charcot, a French neurologist, first named and described this disease (Goetz, 2000). After many decades of research, there is a single treatment available that has been approved to treat ALS patients called riluzole, an anti-glutamatergic drug that has been shown to only marginally extend survival by a few months (Bensimon et al., 1994).

After decades of research, little is known about the specific processes that are involved in motoneuron degeneration and death in ALS. Thus, a great emphasis has been placed worldwide on both uncovering more about the disease and its pathogenesis, as well as on potential avenues for successful treatment options for patients who are suffering from this disease. To these ends, several animal models have been developed and characterized over recent decades. Of these models, the superoxide dismutase 1 (or SOD1) mutant mouse lines have received a great deal of attention.
SOD1-G93A mouse model of motoneuron degeneration

SOD1 mutant lines exhibit a mutation in the superoxidase dismutase 1 gene, which were generated by constitutively expressing mutant human SOD1 mini-genes in mice (Gurney et al., 1994). Of the 15 murine SOD1 mutant models, the G1H (high-copy line with 25 copies) line of the SOD1-G93A mutant murine strain is one of the most widely characterized models of ALS currently available (Turner & Talbot, 2008). Mice overexpressing this form of mutant SOD1 develop a phenotype similar to that of ALS patients including motor impairment, axonal loss, motoneuron death, muscle atrophy and limb weakness (Fischer et al., 2004).

The more aggressive high-copy G93A strain is studied to maximize the extent of motor neuron degeneration throughout disease progression. The early-onset and rapid progression of disease (G1H, with disease onset of 3 months and duration of 1-2 months; (Chiu et al., 1995) contrasts with the relatively latent low-copy G93A strain (G1L, with disease onset of 6-8 months, and duration of 2-3 months; (Gurney, 1997).

Motoneuron Excitability in the SOD1-G93A Model

Electrical and morphological abnormalities affecting neuronal excitability have been noted in G93A mutant motoneurons embryonically (Pieri et al., 2009) and as early as the first week after birth in the mouse (Leroy et al., 2014; Pambo-Pambo et al., 2009; Quinlan et al., 2011; van Zundert et al., 2008; von Lewinski et al., 2008). However, motoneuron death has not been observed until as late as P100 (post-natal day 100) with neuromuscular junction degeneration and functional motor unit loss taking place around
P40 (Fischer et al., 2004; Hegedus et al., 2008). This degeneration is thought to continue to increase until animal death. Thus, alterations in intrinsic motoneuronal excitability may be evident starting early and may continue throughout ALS disease pathogenesis as shown recently (Delestree et al., 2014).

SK Channels and Motoneuron Excitability

Small-conductance potassium channels, or SK channels, are expressed in many excitable cells, including alpha-MNs (Deardorff et al., 2013). Somatic SK channels have been shown to mediate a portion of the after-hyperpolarization (the medium or mAHP) of the action potential in motoneurons (Bayliss et al., 1995; Deardorff et al., 2013; Li & Bennett, 2007; Viana et al., 1993b). The ability to fire repetitive action potentials is modulated by the AHP, which is a period after an action potential where the membrane potential is hyperpolarized, lower than the normal resting membrane potential. The length of the AHP, of which SK channels plays a critical component, would determine the firing frequency of motoneurons. Thus, it has been suggested that regulation of SK channel activity can alter neuronal excitability and that the expression of SK channels may greatly influence intrinsic motoneuronal properties (Deardorff et al., 2013).

One major link between SK channels and regulation of excitability is through calcium. The ionic basis for the neuronal AHP was shown to be an increased potassium conductance induced by an increase in intracellular calcium (Coombs et al., 1955). In motoneurons, N, P/Q-type calcium channels have been implicated as being responsible for the mAHP via the direct activation of SK channels in motoneurons (Bayliss et al.,
1995; Deardorff et al., 2013; Li & Bennett, 2007; Viana et al., 1993b) and both of these channel groups are thought to be spatially clustered away from other calcium sources (Kobayashi et al., 1997; Li & Bennett, 2007).

On alpha-MNs, SK channels, N, P/Q-type calcium channels, voltage-gated potassium channels (Kv), and muscarinic acetylcholine receptors have been shown to be clustered together at cholinergic synapses (Deardorff et al., 2013). Cholinergic activation is thought to inhibit SK channel conductance, leading to a decreased mAHP and an increase in motoneuron firing (Miles et al., 2007). The precise mechanisms behind cholinergic inhibition of SK channels and Kv channels also found at these sites is currently unknown.

**DIAGRAM 1**
SK channels cluster at cholinergic synapses on the soma and proximal dendrites of alpha-MNs. Image adapted from figures from Connaughton et al, 1986; Deardorff et al., 2014; Witts et al., 2013.
The motoneuron mAHP is suppressed or blocked by the elimination, chelation or replacement of calcium (Kobayashi et al., 1997; Lape & Nistri, 2000; Viana et al., 1993a; Zhang & Krmjevic, 1987). Omega-conotoxins, N and P/Q-type channel blockers, effectively block the mAHP in motoneurons comparably to apamin, a direct blocker of SK channels (Kobayashi et al., 1997; Li & Bennett, 2007). Thus, the N, P/Q calcium conductances and the SK conductance are closely linked together.

**Motoneurons Types and SK Channels**

Alpha-MNs can be separated according to the contractile properties of the motor units that they form with three groups of target muscle fibers: fast-twitch fatigable, fast-twitch fatigue-resistant, and slow-twitch fatigue resistant (Burke et al., 1973). Thus, there appear to be at least two fast-type motoneuron groups (FF and FR) and one slow-type motoneuron group (S). In mice, this latter group, identified through electrophysiology, has been shown to selectively express SK3, a specific SK channel isoform (Deardorff et al., 2013). All motoneurons are thought to express SK2, another SK channel isoform (Deardorff et al., 2013).

On the other hand, FF-type motoneurons in the mouse have been identified with other markers including the presence of large amounts of matrix metalloproteinase 9 or MMP9, an enzyme involved in breakdown of extracellular matrix (Kaplan et al., 2014). Motoneurons and the muscle fibers they innervate compose a motor unit, and FF motor units have been identified as being specifically vulnerable to degeneration (Hegedus et al., 2008). Markers for FR-type MNs have yet to be identified.
Maturation of SK channels

Somatic SK channels have been implicated as the primary contributor to the mAHP in neonatal and adult motoneurons (Deardorff et al., 2013; Li & Bennett, 2007; Viana et al., 1993b). Comparisons between neonatal and adult motoneurons implicate the notion that AHP maturation happens over postnatal development (Lape & Nistri, 2000; Li & Bennett, 2007). This maturation in lumbar motoneurons is correlated with matching behavioral changes in locomotion that occur at and after P10, when mice begin to weight-bear and walk (Westerga & Gramsbergen, 1990). Interestingly, it has been suggested that motoneurons in mice are relatively hypoexcitable even at three weeks (roughly P20), with changes in excitability measured via H-reflex occurring thru six weeks of age (Chandran et al., 1991).

Motoneuron AHP in the G93A Model

Quinlan and colleagues reported a decrease in the AHP half-duration in SOD1-G93A mutant neonatal motoneurons using a slice preparation (Quinlan et al., 2011). However, others have seen no change in the AHP properties (Leroy et al., 2014) using the same preparation. Also of interest is that there has been no report of a change in AHP magnitude (Leroy et al., 2014; Quinlan et al., 2011). In the adult G93A mutant mouse, no changes in AHP have been demonstrated (Delestree et al., 2014).

Inhibition of SK channels is putatively suggested as a source of neuromodulation of the AHP and has been shown to affect motor output. AHP modulation has been linked to disease-related changes in the G93A model (Casas et al., 2013; Herron & Miles, 2012;
Saxena et al., 2013). Measures of the AHP (which are mediated partially by the SK) have been demonstrated to be significantly decreased in human patients with ALS (Piotrkiewicz & Hausmanowa-Petrusewicz, 2011). Furthermore, a down regulation of SK (a decrease in SK conductance or \( G_{sk} \)) was predicted in simulations of neonatal mutant SOD1 MNs (Elbasiouny laboratory, unpublished data).

Possible Roles of SK channels in ALS

While SK channels have not been directly implicated in ALS, there has been some evidence suggesting that they may be a viable target for study. Riluzole, the only current treatment for ALS patients, has been shown to activate SK channels (Cao et al., 2002; Grunnet et al., 2001) and this activation has been suggested as the source of therapeutic potential in spinal muscular atrophy, another motoneuron disease (Dimitriadi et al., 2013). In addition, SK2 and SK3 knockout mice exhibit constant tremor, gait disorder, and other abnormal locomotor behaviors (Jacobsen et al., 2008; Szatanik et al., 2008). These could be due to SK effects in alpha-MNs and/or in other neurons that may be involved in motor control.

SK channels via mediating AHP conductance play an important role in regulating MN excitability by controlling firing rate. The elimination of SK conductance through apamin abolishes the mAHP, thereby increasing repetitive firing (Li & Bennett, 2007). Thus, the mAHP is essentially thought to be a blocker of spike generation and a mechanism to reduce firing frequency. Reducing SK conductance could increase or maintain firing frequency in otherwise hypoexcitable cells. Indeed, the post-spike AHP
was reduced in amplitude with increasing firing frequency in cat motoneurons (Kolmodin & Skoglund, 1958). In addition, there is computational evidence that reduction of the AHP may lead to persistent inward current facilitation, leading to further increases in excitability (Elbasiouny et al., 2006).

Also, changes in intracellular calcium have been suggested to play a part in cellular excitotoxicity that may be a major contributor to neurodegeneration. Calcium current enhancement has been linked to ALS pathology (Engelhardt et al., 1995) and N-type channel overexpression on the plasma membrane has been demonstrated in the SOD1-G93A model in cortical neurons from adult mice and from cultured spinal motoneurons (Pieri et al., 2013; Chang & Martin., 2016). In both of these groups of cells, an increase in N-type calcium current was demonstrated and blocking these currents decreased cell mortality in the cortical neuron study.

The N-type current has also been shown to be slowly inactivating in mature spinal motoneurons (Carlin et al., 2000). It is conceivable that a change in calcium channel kinetics via gene splicing or other cellular mechanisms may result in different inactivation speeds (Bourinet et al., 1999; Zhang et al., 1993) leading to changes in the activation of SK channels, which have been shown to be located within 40 nm of their calcium source (Indriati et al., 2013). If SK channel activation is affected in motoneuron degeneration, their expression and clustering properties may be altered.
II. HYPOTHESES
**Hypothesis 1:** Mutant SOD1 motoneurons have altered SK channel clustering that may explain predicted changes in SK channel activity.

*Background*

A shortening of the α-MN mAHP has been noted directly in a mouse *in vitro* electrophysiological study (Quinlan et al., 2011) and indirectly in a study of human muscular electrical activity in ALS patients (Piotrkiewicz & Hausmanowa-Petrusewicz, 2011). Furthermore, modeling data suggest that the mAHP conductance is reduced in mutant SOD1 MNs (Elbasiouny lab, unpublished results). Clustering of SK channels at post-synaptic sites on α-MNs has been shown to be directly responsible for the mAHP conductance (Deardorff et al., 2013). Thus, I hypothesized that SK channel expression would be altered in lumbar mutant SOD1 α-MNs.

*Methods*

Spinal cord immunohistochemistry and quantitative confocal microscopy will be used to determine changes in SK2 and SK3 clustering in lumbar WT and mutant SOD1 α-MNs over disease progression.
**Hypothesis 2:** Selective expression of SK3 is increased over disease progression in mutant SOD1 motoneuron populations.

**Background**

S-type motor units have been suggested to show disease resistance in ALS mouse models (Hegedus et al., 2008). In mice, the motoneurons that innervate these motor units (S-type motoneurons) selectively express SK3-IR (Deardorff et al., 2013). Thus, I hypothesized that selective expression of SK3-IR would increase over disease progression in mutant SOD1 motoneuron populations.

**Methods**

Spinal cord immunohistochemistry and quantitative confocal microscopy will be used to determine changes in selective expression of SK3 in lumbar WT and mutant SOD1 spinal cords over disease progression.
**Hypothesis 3**: Alpha-MN soma size is increased over disease progression in mutant SOD1 spinal cords.

**Background**

Changes in motoneuron size have been demonstrated in neonatal SOD1 mutant mice using electrophysiological and anatomical approaches (Amendola & Durand, 2008; Leroy et al., 2014; Quinlan et al., 2011). These authors and others have suggested the possibility that changes in mutant SOD1 motoneuron size are disease-related. A recent study suggested that mutant SOD1 MNs have an increased soma size at post-natal day 30, demonstrating that motoneuron size changes may exist in adulthood (Shoenfeld et al., 2014). SK3 is putatively expressed in S-type (disease-resistant) MNs and not in F-type (disease-vulnerable) MNs in mice (Deardorff et al., 2013). Thus, I hypothesized that soma size would be increased in mutant SOD1 MNs compared to their WT counterparts, and that increases in soma size would appear selectively in SK3-IR negative motoneurons (F-type).

**Methods**

Spinal cord immunohistochemistry and quantitative confocal microscopy will be used to determine changes in soma size of SK3+ and SK3- motoneurons in lumbar WT and mutant SOD1 spinal cords over disease progression.
III. SPECIFIC METHODS
All procedures were performed according to National Institutes of Health guidelines and approved by the Wright State University Institutional Animal Care and Use Committee. Mice with a B6SJL-Tg genetic background were used for all experiments. Nontransgenic female mice were bred with transgenic male mice expressing either the wild-type human SOD1 gene (SOD1WT) or the human SOD1 gene with a glycine to alanine mutation at amino acid 93 (SOD1G93A). Tail clippings were genotyped for the human SOD1 gene by Transnetyx, Inc. All mutant SOD1 mice expressed a high copy of the mutated gene (>25 copies). Mice were taken down at four time points (postnatal days 10, 30, 90, and end-stage as defined by the Elbasiouny laboratory).

**Animal Surgical Procedures**

All animals were anaesthetized with Euthasol (pentobarbital sodium and phenytoin sodium) solution and transcardially perfused with vascular rinse (0.01 M phosphate buffer with 0.8% NaCl, 0.025% KCl and 0.05% NaHCO₃, pH 7-8) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7-8).

**Tissue Preparation**

The lower lumbar spinal cord was quickly removed and post-fixed in fixative for around 2 hours or overnight. Tissue was then stored in 15% sucrose at 4°C overnight. Transverse sections of L4-L6 spinal cords were then cut on a cryostat at ~50 μm thick, and collected in 0.01 M PBS (pH 7-8). For SK2-IR, tissue was processed with antigen-retrieval procedures. Briefly, tissue was incubated in 10 mM sodium citrate, pH 6.0, with 0.05% Tween 20 at 95°C for 20 min prior to immuno-staining.
**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 cocktails 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, Catalogue# N-21479, Neurotrace, Life Technologies). The SK channel immunoreactivity was localized with polyclonal rabbit anti-SK3 (EMD Millipore, 1:1000 dilution) or polyclonal rabbit anti-SK2 (Alomone, 1:100 dilution) directed against amino acid residues 2–21 of human SK3 or against amino acids 542–559 of SK2. The specificity of these primary antibodies has been described and confirmed with Western blotting by the Fyffe laboratory in their study (Deardorff et al., 2013).

Double labeling was performed by combining one of the anti-SK antibodies with anti-VACHT (vesicular acetylcholine transporter; Abcam, goat, 1:1000 dilution) to restrict analysis to alpha-MNs.

Some slices were immunostained with polyclonal goat anti-MMP9 (Sigma Aldrich, 1:500 dilution), monoclonal mouse anti-NeuN (EMD Millipore, 1:500 dilution), Nissl stain, and anti-SK3. This set of staining was performed following protocols from previous literature (Deardorff et al., 2013; Leroy et al., 2014).

All primary antibodies were diluted in PBS-T 0.1%, pH 7.4, and incubated overnight at 4°C. The sensitivity and specificity of the primary antibodies against the various synaptic proteins and neuronal markers have been previously described by the
Fyffe laboratory and others (Alvarez et al., 1999; Alvarez et al., 1998; Deardorff et al., 2013; Deng & Fyffe, 2004; Muennich & Fyffe, 2004).

Immunoreactivity was detected with species-specific secondary antibodies conjugated to Alexa Fluor 488, Cy3 and Alexa Flour 647 (Jackson Immunoresearch, West Grove, PA, USA) diluted 1:50 in PBS-T 0.1%, pH 7.4, and incubated at room temperature for ~3 h. Sections were then mounted onto slides and cover-slipped in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA).

Confocal imaging, analysis and statistics

Images were obtained on a Fluoview 1000 Olympus (Center Valley, PA, USA) confocal microscope with a ×10 objective at 2.0 μm Z-steps, ×20 objective at 1 μm Z-steps, and ×60 oil immersion objective at 1 μm steps.

Alpha-MNs were differentiated on the basis that soma size measurements fell within a previously published range of >300 μm² (Ishihara et al., 2013) and that they were located in Rexed lamina IX and that they received synaptic input from large cholinergic boutons as evidenced by VACHT-IR. 2-D analysis of MNs was then performed. This process allowed for the relatively efficient analysis of the somatic morphology and neurochemistry of a large sample of MNs from each age and genotype group.

60x Cluster Analysis

Channel cluster dimensions (cluster area and cluster intensity ratios) and cell sizes (largest cross-sectional area) on alpha-MNs were measured with Fluoview software as shown in the image on the following page (Olympus). Regions of interest were drawn
around clusters (as shown by SK3-IR cluster area 1 in Diagram 2) and around the largest cross-sectional area of the soma (not shown). Background intensities were calculated by averaging three randomly sampled areas (areas 9, 10, 11 in Diagram 2) within the neuropil per image analyzed.

Channel cluster areas and intensities were obtained for four time points (P10, P30, P90, and end-stage) in SOD1 mutants and their wild-type littermates. One to eight clusters were analyzed per MNs and their properties were averaged by cell for statistical analysis.

**DIAGRAM 2**
Region of interest drawn around an SK channel cluster (circle 1) and around background regions outside Nissl-stained cells (circles 9, 10, 11).

*60x Density Measurements*

Density measurements were performed according to modified protocols from a previous study (Alvarez et al., 2011). Briefly, Nissl-stained cells stained for VAChT-IR were randomly sampled from both WT and mutant SOD1 neonatal motoneurons. Each cell
was imaged with a separation by 1µm z-steps. From these image stacks a mid-somatic region was identified by the presence of a well-defined nucleolus and from this center image three optical sections separated by 2µm in the z-axis (to avoid sampling the same terminals) were chosen for quantification. The files containing the sections were then labeled with a letter identifier, and the analyzer was blinded to the genotype.

The number of SK3-IR clusters on the surface of VACHT-IR labeled Nissl cell bodies was counted and the largest cellular perimeter (at the mid-somatic region) for each cell was measured, excluding the origins of primary dendrites. Counts were made using ImageJ cell counter software and perimeter measurements were obtained with Fluoview software. Densities were estimated as the number of contacts per 100 µm of linear perimeter. An average density estimate was obtained for each motoneuron.

**20x Proportion Analysis (SK3-IR, VACHT, Nissl)**

Cell sizes (largest cross-sectional area) on putative alpha-MNs (objects positive for VACHT-IR) were measured with Fluoview software (Olympus). Regions of interest were drawn around the largest cross-sectional area of the soma (not shown). MNs were identified as either expressing SK3 (positive) or not expressing SK3 (negative). Care was taken to image and analyze entire motor pools on ventral horn of slices analyzed.

**20x Proportion Analysis (SK3-IR, MMP9-IR, NeuN-IR, Nissl)**

Cell sizes (largest cross-sectional area) on putative alpha-MNs (Nissl-stained objects in Rexed lamina IX within ranges cited prior and positive for NeuN-IR) were measured with Fluoview software (Olympus). Regions of interest were drawn around the
largest cross-sectional area of the soma (not shown). MNs were identified as either expressing SK3 (positive) or not expressing SK3 (negative), and as either expressing MMP9 (positive) or not expressing MMP9 (negative). Care was taken to image and analyze entire motor pools on ventral horn of slices analyzed.

*Image Processing*

For qualitative presentation in thesis, all images were edited first in Fluoview (Olympus software) and enhanced using Adobe Photoshop and Microsoft Word. All images were kept unaltered for quantitative analysis.

*Statistical Analysis*

Statistica and GraphPad Prism 6/7 were used for all statistical analysis. For 60x SK2 and SK3 cluster measurements, two-way ANOVAs followed by Fisher’s LSD and Tukey post-hoc tests were performed to examine effects of genotype and age on cluster size and intensity. For ANOVA analysis, F values are provided to indicate significance of effects. For 20x SK3 expression measurements, Fisher’s exact tests were performed to test for equality of proportions of SK3 expression between WT and mutant SOD1 motoneurons at all ages. For 60x density measurements, a non-parametric Mann-Whitney U test was used to compare between WT and mutant SOD1 motoneurons. For MWU analysis, U values are provided to show the difference between the two rank totals. Significance for all tests was set at $P < 0.05$ or lower.
Table 1

Antibodies/stains used in this thesis.
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<th>ANTIGEN</th>
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IV. RESULTS
**Hypothesis 1:** Mutant SOD1 motoneurons have altered SK channel clustering that may explain predicted changes in SK channel activity.

**Summary of Significant Results:**
Mutant SOD1 MNs appear to have smaller SK-IR clusters compared to their WT counterparts over SOD1 disease progression. Specifically SK2-IR and SK3-IR clusters appear to have a reduced size at four disease time points surveyed (post-natal days 10, 30, 90, and animal end-stage). No changes in cluster intensity were observed between WT and mutant SOD1 MNs at any time points. SK3-IR cluster density was found to be reduced in neonatal mutant SOD1 motoneurons compared to their WT counterparts. SK2-IR cluster sizes were found to be smaller in putative F-type motoneurons (LCA >1100 sq. microns, established through quantitative measurements) in mutant SOD1 mice as compared to their WT counterparts at two adult time points.

**SK2 and SK3 immunoreactivity in WT and mutant SOD1 MNs**

SK2 and SK3 immunoreactivities (SK2-IR and SK3-IR) were compared in the ventral horn of spinal cords from WT and mutant SOD1 mice at various stages of development. A number of time points were analyzed [10 days (P10), 30 days (P30), 90 days (P90), and animal end-stage (ES)] to determine the time course of SK expression, and to analyze possible changes resulting from ALS disease pathogenesis.

The postnatal age P10 was chosen to study an early neonatal time-point at which electrical abnormalities in this model have been observed (Quinlan et al., 2011). In addition, SK channel activity was predicted to be downregulated at this time point.
(Elbasiouny laboratory, unpublished data). The P30 and P90 time points were chosen as points in which all mice had advanced in development, and at early and late stages of disease progression respectively. Early signs of motoneuron denervation (putatively FF-type MNs) have been detected after P30 with a large decline in motoneurons around P90 (putatively S-type MNs) in this model (Hegedus et al., 2008). Animals showing full hind-limb analysis (failure of righting test under Elbasiouny laboratory protocols) were sacrificed for analysis of alpha-MNs at animal end-stage.

One to eight spinal cord sections of SK2 and SK3 per animal were analyzed (n = 1 – 8) from 3-5 animals per age per genotype (N = 3-5). SK2 and SK3 channels appear to be expressed in large clusters (area > 1 µm²) in both WT and mutant SOD1 motoneurons of all ages. Figures 1-4 show representative histological images from lumbar spinal cords in both WT and mutant SOD1 animals, in which VAChT-IR labeled motoneurons expressing SK3-IR and SK2-IR.

Qualitative analysis of 60x confocal images of alpha-MNs as identified by VAChT-IR expression indicate that SK2-IR expression is largely present in most WT and mutant SOD1 alpha-MNs. SK3-IR appears to be present only in a subset of alpha-MNs in the spinal cord as observed by a previous study (Deardorff et al., 2013).
FIGURE 1
SK3-IR is expressed in clusters at cholinergic synapses in a subset of WT motoneurons at all ages of spinal cord development.
Low magnification (20x) of a representative histological section from the lower lumbar spinal cord in an adult WT mouse. (A) Labeling of VACHT-IR identifies alpha-MNs in ventral horn of spinal cord. (B) Co-localization of VACHT-IR and SK3-IR shows synaptic location of SK3 channel expression. (C-D) SK3-IR expression in a subset of motoneurons. Scale bar is 10 μm. Red arrows indicate SK3-IR and the white arrows indicate an SK3-IR negative motoneuron.
FIGURE 1

WT
FIGURE 2
SK3-IR clusters at the cholinergic sites are still observable in a subset of mutant SOD1 motoneurons at all ages of spinal cord development.
Low magnification (20x) of a representative histological section from the lower lumbar spinal cord in an adult mutant SOD1 mouse. (A) Labeling of VACHT-IR identifies alpha-MNs in ventral horn of spinal cord. (B) Co-localization of VACHT-IR and SK3-IR shows synaptic location of SK3 channel expression. (C-D) SK3-IR expression in a subset of motoneurons. Scale bar is 10 μm. Red arrows indicate SK3-IR and the white arrows indicate an SK3-IR negative motoneuron.
FIGURE 2

mutant SOD1
FIGURE 3
SK2-IR is expressed in post-synaptic clusters at cholinergic synapses in a vast majority of WT motoneurons at all ages of spinal cord development.
Low magnification (20x) of a representative histological section from the lower lumbar spinal cord in an adult WT mouse. (A) Labeling of VAChT-IR identifies alpha-MNs in ventral horn of spinal cord. (B) Co-localization of VAChT-IR and SK2-IR shows synaptic location of SK2 channel expression. (C-D) SK2-IR expression is seen in many motoneurons. Scale bar is 10 μm. Arrows indicate SK2-IR.
FIGURE 3

WT
FIGURE 4
SK2-IR clusters continue to appear at cholinergic synapses on mutant SOD1 motoneurons at all ages of spinal cord development.
Low magnification (20x) of a representative histological section from the lower lumbar spinal cord in an adult mutant SOD1 mouse. (A) Labeling of VACHT-IR identifies alpha-MNs in ventral horn of spinal cord. (B) Co-localization of VACHT-IR and SK2-IR shows synaptic location of SK2 channel expression. (C-D) SK2-IR expression is seen in many motoneurons. Scale bar is 10 μm. Arrows indicate SK2-IR.
FIGURE 4

mutant SOD1
**SK2 cluster size changes in WT and mutant SOD1 MNs**

Quantitative analysis of SK2-IR cluster properties revealed significant differences in cluster size. Figure 5 shows analysis of motoneuron clusters in spinal cord slices stained for SK2-IR, which revealed that SK2 clusters are smaller in mutant SOD1 motoneurons in adult mice compared to their WT counterparts. The mean largest cross-sectional area of SK2 channel clusters in WT lumbar alpha-MNs is 7.07, 9.34, 8.5, and 8.47 μm² compared to 6.68, 8.5, 7.24, and 7.24 μm² in mutant SOD1 motoneurons at P10, P30, P90, and ES time points respectively. A two-way ANOVA was used to examine the effects of both genotype and age on cluster surface area. Significant effects of genotype and age were revealed [F (1,265) = 29.17; p<0.0001] and [F (3,265) = 22.32; (p<0.0001)], with no interaction effect, showing that genotype and age both had an effect on SK2 cluster size.

Post-hoc analysis with Fisher’s Least Significant Difference revealed significant decreases in SK2 cluster size at P30, P90 and ES between WT and mutant SOD1 motoneurons. Significant increases in cluster size were also observed between motoneurons at P10 and P30 with motoneurons from later time points in both WT and mutant SOD1 mice, as shown in Figure 5B-C. Tukey’s post-hoc analysis was also conducted in order to account for multiple comparisons. This post-hoc analysis confirmed the significant differences between WT and mutant SOD1 motoneurons from P90 and ES mice.
Another notable difference was detected between P10 and the other time points in WT motoneurons. Clusters appeared to increase in size between P10 and the older time points, suggesting a possible increase in SK2-IR expression in older motoneurons.

**SK2 cluster intensity changes in WT and mutant SOD1 MNs**

Analysis of SK2-IR cluster intensity revealed no significant differences between WT and mutant SOD1 motoneurons. Figure 6 shows the average cluster intensity differences between WT and mutant SOD1 motoneurons at the four time points analyzed. The mean intensity (intensity/background ratio) of SK2 channel clusters in WT lumbar alpha-MNs is 1.36, 1.49, 1.64, and 1.63 compared to 1.41, 1.49, 1.66, and 1.53 in mutant SOD1 motoneurons at P10, P30, P90, and ES time points respectively. A two-way ANOVA was used to examine the effects of both genotype and age on cluster intensity. A significant effect due to age was revealed \( [F (3,264) = 15.26; p<0.0001] \), with no genotype or interaction effect, showing that SK2 cluster intensity increased over age.

To examine changes in SK2-IR cluster intensity over time in WT mice, SK2-IR cluster intensity was compared between each time point and the one following it (P10 vs P30, P30 vs P90, etc) with post-hoc analysis. This revealed significant increases in SK2-IR cluster intensity over time in WT mice. To examine changes in SK2-IR cluster intensity over time in mutant SOD1 mice, SK2-IR cluster intensity in mutant SOD1 MNs was compared between each time point and the one following it (P10 vs P30, P30 vs P90, etc) with post-hoc analysis. This also revealed significant increases in mutant SOD1
mice. Taken together, these results indicate the maturation of SK2-IR in motoneurons over time. All SK2-IR post-hoc effects are noted in Figures 5 and 6.
FIGURE 5
SK2-IR clusters are smaller in mutant SOD1 motoneurons compared to their WT counterparts.

(A) Average SK2-IR cluster areas (in square microns) of WT (white) and mutant SOD1 (black) motoneurons at four time points are shown, with genotype post-hoc effects of ANOVA performed noted. The number of MNs in each group is noted inside each bar, and significance of post-hoc test is indicated with appropriate symbols (*/T, **/TT, ***/TTT, ****/TTTT for p < 0.05, 0.01, 0.001, 0.0001 respectively).

(B-C) Post-hoc effects for ANOVAs performed on WT and SOD MN SK2-IR cluster area. All effects are using Tukey’s post-hoc unless otherwise noted (FLSD signifies Fisher’s LSD) and significance is noted as follows: * (p < 0.05), ** (p < 0.01), *** (p < 0.001), and **** (p < 0.0001).
FIGURE 5

A

![Bar chart showing average cluster area (sq microns) for WT and SOD groups across P10, P30, P90, and ES stages.]

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FIGURE 6
No changes in SK2-IR cluster intensity were detected between mutant SOD1 and WT motoneurons.
(A) Average SK2-IR cluster intensity ratios (intensity over average background) of WT (white) and mutant SOD1 (black) motoneurons at four time points are shown, with genotype post-hoc effects of ANOVA performed noted. The number of MNs in each group is noted inside each bar, and significance of post-hoc test is indicated with appropriate symbols (*/T, **/TT, ***/TTT, ****/TTTT for p < 0.05, 0.01, 0.001, 0.0001 respectively).
(B-C) Post-hoc effects for ANOVAs performed on WT and SOD MN SK2-IR cluster intensity. All effects are using Tukey’s post-hoc unless otherwise noted (FLSD signifies Fisher’s LSD) and significance is noted as follows: * (p < 0.05), ** (p < 0.01), *** (p < 0.001), and **** (p < 0.0001).
FIGURE 6

A

![Graph showing average cluster intensity for WT and SOD clusters across different time points: P10, P30, P90, and ES. The graph includes error bars and indicates statistical significance with symbols.

B

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**SOD CLUSTER**

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**SK3 cluster size changes in WT and mutant SOD1 MNs**

Quantitative analysis of SK3-IR cluster properties also revealed significant differences in cluster size. Figure 7 shows analysis of motoneuron clusters in spinal cord slices stained for SK3-IR, which revealed that SK3 clusters are smaller in mutant SOD1 motoneurons in neonatal and adult mice compared to their WT counterparts. The mean surface area of SK3 channel clusters in WT lumbar alpha-MNs is 8.49, 8.38, 9.45, and 8.83 μm² compared to 6.32, 7.15, 7.72, and 6.96 μm² in mutant SOD1 motoneurons at P10, P30, P90, and ES time points respectively. A two-way ANOVA was used to examine the effects of both genotype and age on cluster surface area. Significant effects of genotype and age were revealed [F (1,265) = 53.43; p<0.0001] and [F (3,265) = 4.295; (p=0.0056)], with no interaction effect.

To examine changes in SK3 cluster area over time in WT mice, SK3-IR cluster area was compared between each time point and the one following it (P10 vs P30, P30 vs P90, etc) with post-hoc analysis. This revealed no significant changes in SK3-IR cluster area in WT mice. To examine changes in SK3-IR cluster area over time in mutant SOD1 mice, SK3-IR cluster area in mutant SOD1 MNs was compared between each time point and the one following it (P10 vs P30, P30 vs P90, etc) with post-hoc analysis. This also revealed no significant changes in mutant SOD1 mice. All SK3-IR post-hoc effects are noted in Figures 7 and 8.
Analysis of SK3-IR cluster intensity revealed no significant differences between WT and mutant SOD1 motoneurons. Figure 8 shows the average cluster intensity differences between WT and mutant SOD1 motoneurons at the four time points analyzed. The mean intensity (intensity/background ratio) of SK3 channel clusters in WT lumbar alpha-MNs is 1.78, 1.92, 2.55, and 2.19 compared to 1.79, 2.23, 2.48, and 2.38 in mutant SOD1 motoneurons at P10, P30, P90, and ES time points respectively. A two-way ANOVA was used to determine the effects of both genotype and age on cluster intensity. A significant effect due to age was revealed \[ F(3,265) = 21.17; p<0.0001 \], with no genotype or interaction effect.

To examine changes in SK3 cluster intensity over time in WT mice, SK3-IR cluster intensity was compared between each time point and the one following it (P10 vs P30, P30 vs P90, etc) with post-hoc analysis. This revealed a significant increase in SK3-IR cluster intensity after P30 in WT mice. To examine changes in SK3-IR cluster intensity over time in mutant SOD1 mice, SK3-IR cluster intensity in mutant SOD1 MNs was compared between each time point and the one following it (P10 vs P30, P30 vs P90, etc) with post-hoc analysis. This revealed a significant increase in SK3-IR cluster intensity after P10 in mutant SOD1 mice. The difference in SK3-IR intensity change over time (after P30 for WT, after P10 for mutant SOD1 MNs) indicates a possible change in SK3-IR cluster maturation for mutant SOD1 MNs. All SK3-IR post-hoc effects are noted in Figures 7 and 8. Both SK2- and SK3-IR clusters appear to be less pronounced in expression in alpha-MNs from neonatal mice (Figures 6 and 8).
FIGURE 7
SK3-IR clusters are smaller in mutant SOD1 motoneurons compared to their WT counterparts.

(A) Average SK3-IR cluster areas (in square microns) of WT (white) and mutant SOD1 (black) motoneurons at four time points are shown, with genotype post-hoc effects of ANOVA performed noted. The number of MNs in each group is noted inside each bar, and significance of post-hoc test is indicated with appropriate symbols (*/T, **/TT, ***/TTT, ****/TTTT for p < 0.05, 0.01, 0.001, 0.0001 respectively).

(B-C) Post-hoc effects for ANOVAs performed on WT and SOD MN SK3-IR cluster area. All effects are using Tukey’s post-hoc unless otherwise noted (FLSD signifies Fisher’s LSD) and significance is noted as follows: * (p < 0.05, ** (p < 0.01), *** (p <0.001), and **** (p < 0.0001).
FIGURE 7

A

![Bar chart showing average cluster area (sq microns) for WT and SOD clusters across different stages: P10, P30, P90, and ES.](chart_image)

B

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FIGURE 8
No changes in SK3-IR cluster intensity were detected between mutant SOD1 and WT motoneurons.
(A) Average SK2-IR cluster intensity ratios (intensity over average background) of WT (white) and mutant SOD1 (black) motoneurons at four time points are shown, with genotype post-hoc effects of ANOVA performed noted. The number of MNs in each group is noted inside each bar, and significance of post-hoc test is indicated with appropriate symbols (*/T, **/TT, ***/TTT, ****/TTTT for p < 0.05, 0.01, 0.001, 0.0001 respectively).
(B-C) Post-hoc effects for ANOVAs performed on WT and SOD MN SK3-IR cluster intensity. All effects are using Tukey’s post-hoc unless otherwise noted (FLSD signifies Fisher’s LSD) and significance is noted as follows: * (p < 0.05), ** (p < 0.01), *** (p <0.001), and **** (p < 0.0001).
FIGURE 8

A

![Average Cluster Intensity (Intensity/Background)]

B

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46
SK cluster density changes in WT and mutant SOD1 MNs

Following the observation of reduced SK cluster sizes in mutant SOD1 motoneurons, cluster density measurements were undertaken to account for possible compensatory effects in clustering. Density analysis was limited to SK3-IR as analysis of SK2-IR cluster density was qualitatively assessed to be error-prone due to low intensity over background. Figure 9 shows the outcome of SK3 cluster density measurements from analysis performed on neonatal motoneurons. A cluster count of SK3-IR per 100 µm of both WT and mutant SOD1 motoneurons revealed a significant decrease in cluster density in mutant SOD1 motoneurons (p < 0.05, Mann-Whitney U test, U = 168). The median value for WT cluster density was 3.398 per 100 µm (n=22 MNs from 3 animals) compared to 2.78 per 100 µm (n= 25 MNs from 3 animals), showing an 18 percent decrease in the mutant SOD1 MNs. This indicates that there may be less SK clusters on mutant SOD1 MNs.
FIGURE 9
SK3 cluster density is reduced in neonatal mutant SOD1 motoneurons compared to their WT counterparts.
A box and whisker plot of SK3-IR cluster density values (cluster counts per 100µm) of WT and mutant SOD1 neonatal motoneurons (P10) is shown, with significance due to Mann-Whitney U test noted with * (p<0.05).
FIGURE 9

P10 SK Cluster Density

Cluster count per 100 um

WT  SOD
Effects of Soma Size on SK Clustering in Neonatal MNs

After observing the consistent cluster size changes in WT and SOD1 MNs, the possible effects of soma size on this parameters was investigated to determine if MNs of different sizes experienced differing changes in cluster size. Largest cross-sectional area measurements were obtained for every cell analyzed. Lines of regression were plotted, as shown in Figures 10 and 11, and Pearson r values were calculated for WT and mutant SOD1 lines in SK2 and SK3-IR motoneurons. For SK2, r values were 0.65, 0.58, 0.35, and 0.27 in WT mice compared to -0.11, 0.3, 0.11, and 0.52 in SOD mice at P10, P30, P90, and ES respectively. For SK3, r values were 0.88, 0.5, 0.56, and 0.59 in WT mice compared to 0.54, 0.57, 0.4, and 0.69 in SOD mice at P10, P30, P90, and ES respectively.

Linear regression analysis was performed on all MN at every time point to examine the overall effect of soma size on SK2-IR and SK3-IR cluster area on WT and SOD cells. In figure 10, the slopes of WT and SOD SK2-IR cluster area regression lines were shown to be significantly different at P10 (F = 11.4627, p = 0.0013), with the slope of both the WT and SOD lines shown to be significantly non-zero (p< 0.0001 and p=0.0007). The slopes of WT and SOD SK3-IR cluster area regression lines were shown to be significantly different at P10 (F = 11.7155, p = 0.001), with only the slope of the WT line shown to be significantly non-zero (p < 0.0001). Taken together, this indicates that there are differences in cluster size related to soma size between WT and mutant SOD1 MNs.
FIGURE 10
Changes in slope were detected between neonatal (P10) WT and mutant SOD1 motoneuron regression lines of SK2 and SK3 cluster areas vs. soma sizes. Scatterplots of average SK2-IR and SK3-IR cluster areas (in square microns) of WT (blue) and mutant SOD1 (red) motoneurons at P10 are shown, with significant changes in slope from linear regression analysis noted in red (π for p < 0.05).
FIGURE 10

SK2 Cluster Size

Soma Size (µm²)  Cluster Area (µm²)  
300  800  1100  1600

Slope: IT

SK3 Cluster Size

Soma Size (µm²)  Cluster Area (µm²)  
300  800  1100  1600

Slope: IT

WT  SOD
As shown in Figure 11, the slopes of WT and SOD SK2-IR cluster area regression lines were not significantly different at any of the adult time points, with the slope of only the WT line shown to be significantly non-zero (p = 0.0004; p = 0.0336; p = 0.042). The slopes of WT and SOD SK3-IR cluster area regression lines were not significantly different at any of the adult time points, with both the WT and SOD lines shown to be significantly non-zero (P = 0.0003 and p = 0.0039; p = 0.0005 and p = 0.0227; p = 0.0003 and p < 0.0001). The significance of the non-zero lines indicate that there is a linear relationship between soma size and cluster size only in the WT MNs. While the slopes were not different, there was a genotype difference in intercepts in both SK2-IR and SK3-IR regression lines for P30 and P90 MNs (F = 5.566, p = 0.0215; F = 16.42, p = 0.0001) and (F = 12.97, p = 0.0006; F = 20.64, p < 0.0001) and only in the SK3-IR regression line for ES MNs (F = 9.603, p = 0.0029).
FIGURE 11
Changes in intercept were detected between adult WT and mutant SOD1 motoneuron regression lines of SK2 and SK3 cluster areas vs. soma sizes. Scatterplots of average SK2-IR and SK3-IR cluster areas (in square microns) of WT (blue) and mutant SOD1 (red) motoneurons at P30, P90, and ES are shown, with significant changes in intercepts from linear regression analysis noted in red (π for p < 0.05, ππ for p < 0.01, and πππ for p <0.001).
FIGURE 11

SK2 Cluster Size

P30

Cluster Area (um²) vs. Soma Size (um²)

P90

Cluster Area (um²) vs. Soma Size (um²)

ES

Cluster Area (um²) vs. Soma Size (um²)

WT

SOD
Identification of MN types

Possible co-expression of SK3-IR and MMP9-IR (FF-type marker) was studied to confirm absence of SK3-IR in F-type MNs, as suggested by previous literature (Deardorff et al., 2013). Briefly, adult WT (P120+) spinal cord slices were stained with Nissl NeuroTrace, anti-SK3, anti-MMP9, and anti-NeuN antibodies. In adult WT MNs, the putative S-type MN marker SK3-IR was found to be present in a group of putative alpha-MNs that did not express the FF-type MN marker MMP9-IR (Figure 12). A total of 234 cells from whole spinal cord slice samples were sampled from two WT mice for quantitative analysis of SK3 and MMP9 staining. Interestingly, over 95 percent of NeuN-expressing MNs with a LCA (largest cross-sectional area) over 1100 sq. microns (a total of 52 cells) were SK3-IR negative (Figure 12). 80 percent of these SK3-IR negative cells strongly expressed MMP9-IR. Also of note was the qualitative observation that SK3-IR positive cells appeared to weakly express NeuN-IR as compared to MMP9-IR positive cells, as suggested by previous literature (Deardorff et al., 2013).

Furthermore, while SK3-IR (15 percent of the total population) and MMP9-IR (50.4 percent of the population) were never co-expressed, there was a third large group of NeuN-IR cells that did not express either SK3-IR or MMP9-IR (34.6 percent of the total population). This suggested that even with a significant overlap in soma size ranges of slow and fast-type motoneurons, there is at least a soma size range in adult MNs (>1100 sq. microns) at which almost all cells are F-type MNs (and a majority are FF-type). In addition, there appear to be at least two putative groups of SK3-IR negative MNs, one of which strongly expresses MMP9-IR.
FIGURE 12
SK3-IR and MMP9-IR are expressed in different subgroups of NeuN-IR positive alpha-MNs in adult WT spinal cords.
Low magnification (20x) of a representative histological section from the lower lumbar spinal cord in an adult WT mouse. (A) Labeling of NeuN-IR in Rexed lamina IX identifies putative alpha-MNs in ventral horn of spinal cord. (B) A subset of cells from same section express strong MMP9-IR and NeuN-IR (not shown) (C) SK3-IR expression is found in a subset of motoneurons also expressing NeuN-IR (D) SK3-IR and MMP9-IR are not found in same subgroup of cells. (E) Histogram of soma sizes of putative alpha-MNs showing presence or absence of SK3 and MMP9 in WT lower lumbar sections. Scale bar is 30 μm.
FIGURE 12

WT Alpha-MN Soma Size By Putative Type

Largest Cross-Sectional Area (sq microns)

Percent of obs

MMP9+/SK3-
MMP9-/SK3-
MMP9-/SK3+

WT Alpha-MN Soma Size By Putative Type

Percent of obs

Largest Cross-Sectional Area (sq microns)
SK2 Cluster Differences in F-type WT and mutant SOD1 MNs

Following the observation that almost all motoneurons with an LCA greater than 110 sq. microns were putatively F-type (SK3-IR negative), SK2-IR cluster size comparisons were made between putative F-type motoneurons in WT and mutant SOD1 mice at P30 and P90. No comparisons meeting the qualification (motoneurons with a LCA greater than 1100 sq. microns present in both groups) were available at the P10 (neonatal) or ES (end-stage) time points. Figure 13 shows the outcome of SK2-IR cluster size measurements from analysis performed on these SK3-IR negative motoneurons at P30 and P90.

A significant decrease in cluster size in putative F-type mutant SOD1 motoneurons compared to their WT counterparts was revealed at P30 (p = 0.007, Mann-Whitney U test, U = 15). The median value for WT SK2-IR cluster size was 10.91 sq. microns (n=10 MNs from 4 animals) compared to a SOD SK2-IR cluster size of 9.2 sq. microns (n= 10 MNs from 3 animals).

A significant decrease in cluster size in putative F-type mutant SOD1 motoneurons compared to their WT counterparts was revealed at P90 (p = 0.011, Mann-Whitney U test, U = 12). The median value for WT SK2-IR cluster size was 10.33 sq. microns (n=9 MNs from 2 animals) compared to a SOD SK2-IR cluster size of 7.96 sq. microns (n= 9 MNs from 3 animals).
FIGURE 13
SK2-IR cluster size is decreased in mutant SOD1 F-type MN (soma LCA greater than 1100 sq. microns) compared to their WT counterparts at P30 and P90 (adult time-points). Scatter plots of SK2-IR cluster area values (in square microns) of WT and mutant SOD1 motoneurons at P30 and P90 are shown, with significance due to Mann-Whitney U test noted with * (p<0.05) and ** (p<0.01).
FIGURE 13

P30 F-type MN
SK2 Cluster Size

Cluster Area (m^2)

Genotype

WT
SOD

**

P90 F-type MN
SK2 Cluster Size

Cluster Area (m^2)

Genotype

WT
SOD

*
**Hypothesis 2:** Selective expression of SK3 is increased over disease progression in mutant SOD1 motoneuron populations.

*Summary of Significant Results:*

Mutant SOD1 spinal cords appear to have a larger proportion of SK3+ MNs in late adult stages of disease progression. However, an unexpected reduction in SK3+ MNs is seen in early adult mutant SOD1 mice. Furthermore, selective expression of SK3-IR appears to be significantly higher in mice through the first month of life than in later adulthood.

*Proportions of SK3-expressing MNs in WT and mutant SOD1 spinal cords*

The proportion of SK3-expressing MNs in the lumbar spinal cords decreases over development in both WT and mutant SOD1 motoneurons, but increases at end-stage of disease progression. Figure 14 shows pie charts documenting the proportions of WT and mutant SOD1 motoneurons expressing SK3 over time (P10, P30, P90, and ES).

In WT MN development, the proportion of cells that do not express SK3 (SK3-IR negative cells) increases over time (from P10 to P90) and becomes steady in adulthood (53% at P10, 71% at P30, 86% at P90, and 84% at ES, as shown in Figure 14). In contrast, in mutant SOD1 MN development, the proportion of cells that are SK3-IR negative increases early and then decreases over time (60% at P10, 81% at P30, 76% at P90, and 42% at ES, as shown in Figure 14). Fisher's exact tests were used to determine if proportions of SK3 expression were variable between genotypes. All of the adult time
points showed significant differences between WT and mutant SOD1 slice expression of SK3.

At P30, there was a significant decrease in the expression of SK3 in mutant SOD1 slices compared to their WT counterparts (p = 0.0058). Interestingly, at P90, there was a significant increase in the expression of SK3 in mutant SOD1 slices compared to their WT counterparts (p = 0.0023). At ES, there was also a significant increase in the expression of SK3 in mutant SOD1 slices compared to their WT counterparts (p < 0.0001).
FIGURE 14
The number of alpha-MNs expressing SK3 decreases over development in WT and mutant SOD1 spinal cords.
Pie graphs of SK3 expression proportions in WT (blue) and mutant SOD1 (red) spinal cord slices at P10, P30, P90, and ES is shown, with significant changes as detected by Fisher’s exact test noted with * (p < 0.05), ** (p < 0.01), *** (p < 0.001), or **** (p < 0.0001). Inset boxes provide percentages of proportions of MNs in spinal cord slices expressing or not expressing SK3 and the number of MNs sampled in each group is noted below each corresponding circle. Three or more animals per group were included for all SK3-expression analysis at all time points.
FIGURE 14
**Hypothesis 3:** Alpha-MN soma size is increased over disease progression in mutant SOD1 spinal cords.

**Summary of Significant Results:**

Soma size appears to be increased in mutant SOD1 MNs until animal end-stage, where it is significantly reduced. The changes appear to be significant only in SK3- MNs and reverse at disease end-stage.

**Soma size comparisons between WT and mutant SOD1 motoneurons**

SK3-IR expression was used as a marker for soma size analysis of motoneurons from WT and mutant SOD1 spinal cords at P10, P30, P90, and ES time points. Briefly, the number of SK3-IR expressing MNs was studied in transverse lower lumbar spinal sections (L4-L6) in WT and mutant SOD1 mice at each time point. WT and SOD soma sizes were compared at each time point as shown in Figure 15. A two-way ANOVA was used to determine significant differences, with Tukey post-hoc tests to highlight any potential source of differences detected. At P10, there were significant effects due to genotype [WT vs SOD, $F(1,609) = 8.2661, p = 0.00418$] and SK3-IR expression [SK3- vs SK3-, $F(1,609) = 50.746, p = .000001$] on largest cross-sectional area measures, with no interaction effect. Post hoc analysis revealed that SK3-IR negative motoneurons had a significantly larger mean soma size than SK3-IR positive motoneurons in both WT (a mean of 756.59 +/- SEM of 15.56 sq. microns in SK3- MNs compared to a mean of 637.08 +/- SEM of 15.07 sq. microns in SK3+ MNs) and mutant SOD1 motoneurons (a mean of 838.23 +/- SEM of 20.57 sq. microns in SK3- MNs compared to a mean of 838.23 +/- SEM of 20.57 sq. microns in SK3- MNs compared to a mean of
671.12 ± SEM of 21.49 sq. microns in SK3+ MNs) (p = 0.0051 and p = 0.000008). In addition, SK3-IR negative mutant SOD1 motoneurons were significantly larger than their WT counterparts (p = 0.012).

At P30, there were significant effects due to genotype [WT vs SOD, F (1,606) = 14.093, p = 0.00019] and SK3-IR expression [SK3+ vs SK3-, F (1,606) = 77.693, p = .00001] on largest cross-sectional area measures, with no interaction effect. Post hoc analysis revealed that SK3-IR negative motoneurons had a significantly larger mean soma size than SK3-IR positive motoneurons in both WT (a mean of 846.66 ± SEM of 19.98 sq. microns in SK3- MNs compared to a mean of 611.16 ± SEM of 26.61 sq. microns in SK3+ MNs) and mutant SOD1 motoneurons (a mean of 931.56 ± SEM of 16.57 sq. microns in SK3- MNs compared to a mean of 717.67 ± SEM of 28.34 sq. microns in SK3+ MNs) (p = 0.000008 and p = 0.000008). In addition, SK3-IR negative mutant SOD1 motoneurons were significantly larger than their WT counterparts (p = 0.0037).

At P90, there was a significant effect due to SK3-IR expression [SK3+ vs SK3-, F (1,617) = 36.189, p = .000001] on largest cross-sectional area measures, with no genotype or interaction effect. Post hoc analysis revealed that SK3-IR negative motoneurons had a significantly larger mean soma size than SK3-IR positive motoneurons in both WT (a mean of 828.14 ± SEM of 15.59 sq. microns in SK3- MNs compared to a mean of 643.73 ± SEM of 26.23 sq. microns in SK3+ MNs) and mutant SOD1 motoneurons (a mean of 823.45 ± SEM of 14.35 sq. microns in SK3- MNs compared to a mean of 712.52 ± SEM of 26.3 sq. microns in SK3+ MNs) (p = 0.000019 and p = 0.00131).
At ES, there were significant effects due to SK3-IR expression [SK3+ vs SK3-, F (1,545) = 11.867, p = .00062] and genotype expression [WT vs SOD, F (1,545) = 155.28, p = .00001] on largest cross-sectional area measures. In addition, there was an interaction effect [F (1,545) = 5.4379, p = .02]. Post hoc analysis revealed that SK3-IR negative motoneurons had a significantly larger mean soma size than SK3-IR positive motoneurons only in WT (a mean of 919.92 +/- SEM of 20.65 sq. microns in SK3- MNs compared to a mean of 766.04 +/- SEM of 33.87 sq. microns in SK3+ MNs) motoneurons (p = 0.0011). In addition, SK3-IR negative mutant SOD1 motoneurons (a mean of 528.09 +/- SEM of 19.1 sq. microns) were significantly smaller than their WT counterparts (p = 0.000008) and SK3-IR positive mutant SOD1 motoneurons (a mean of 498.72 +/- SEM of 12.34 sq. microns) were significantly smaller than their WT counterparts (p = 0.000008).
FIGURE 15
An enlargement of soma size is detected in early stages of development and disease progression in SK3-IR negative mutant SOD1 motoneurons. Bar graphs of largest cross-sectional areas (in square microns) of SK3-IR positive (shaded) and negative (solid) MNs from WT (blue) and mutant SOD1 (red) spinal cord slices at P10, P30, P90, and ES are shown. Significant changes as detected by Tukey’s post-hoc following an ANOVA examining the effects of SK3 expression and genotype on soma size are noted with * (p < 0.05), ** (p < 0.01), *** (p < 0.001), or **** (p < 0.0001).
V. DISCUSSION
SK channel clustering is altered in SOD1 mutant MNs

SK channel clusters were shown to be smaller in SOD1 mutants from all time points surveyed. Specifically, smaller SK3 cluster sizes were detected in motoneurons throughout the mouse lifespan, while SK2 cluster sizes were smaller in adult mice. The changes in SK2 cluster sizes were also noted specifically in the SK3-IR negative (putative F-type) population of motoneurons. In addition, the number of cells expressing SK3 appears to be reduced early on in the SOD1 disease progression. Changing SK expression could have a major effect on reducing neuronal SK conductance as hypothesized, and therefore play a large role in altering motoneuron AHP.

It is possible that the SK channel cluster size is reduced to balance a putative N-type calcium current increase in motoneurons as elevated cytosolic calcium might reduce MN excitability through SK activation. The relatively small changes in SK cluster size, in comparison to changes seen after nerve injury (Deardorff, 2015), that we are reporting support this theory of fine tuning on the part of the cell to maintain net AHP parameters. On the other hand, the decrease in cluster density in combination with these cluster size changes indicates a larger phenomenon in decreasing SK expression. How these putative changes in SK expression could affect the AHP conductance in motoneurons through SOD1 disease progression remains unanswered.

It was recently demonstrated that dendritic calcium transients appear to be reduced in neonatal mutant SOD1 motoneurons (Quinlan et al., 2015). Although not directly related, it is possible that somatic calcium transients are also reduced. Thus, it could be that the reduced expression of SK channels is part of a larger cellular push to
increase MN excitability, partly by down regulating the AHP conductance. This is supported by the significant increase in soma size of neonatal and P30 putative F-type (SK3-IR negative) motoneurons as well as the reduced SK3 cluster density in neonatal motoneurons, which would both lead to reduction in SK expression per unit area in motoneurons.

The literature implicating a reduced AHP (Quinlan et al, 2011) in these cells and the simulations suggesting a decreased SK conductance in SOD-like motoneurons (Elbasiouny laboratory, unpublished data) further support this theory. Additionally, there has been postulation that motoneurons become hypoexcitable later on in ALS disease progression (Delestree et al., 2014; Devlin et al., 2015; Wainger et al., 2014). Thus, there may be global mechanisms involved in restoring cell excitability that may include changes in SK channel expression, thereby affecting SK conductance and the mAHP.

Modeling has shown that the AHP amplitude in MNs can be adjusted by varying the density of potassium channels, changing the density of calcium channels or both, while the AHP duration can only be adjusted by changing calcium activated potassium channel parameters (Vieira & Kohn, 2007). This fits nicely with data that suggests that the mAHP duration is shortened eight days following tibial nerve crush, likely via a reduction in SK3 cluster area (Deardorff, 2015). Thus, the cluster changes that are shown here implicate a probable physiological effect on AHP half-duration.

However, there remains a corollary possibility that a mutation-related change in SK channel expression in turn causes a downstream putative up regulation in N-type calcium current. This seems reasonable given evidence suggesting the role of chaperone proteins like Sigma1Rs in SK channel trafficking and in ALS pathogenesis (Mavlyutov et
It has been shown that Sigma1R may play a role in increasing the activity of SK channels, either directly or via calcium activation (Mavlyutov et al., 2012).

Interestingly, the absence of S1R was shown to increase MN excitability and exacerbate disease progression in a G93A model (Mavlyutov et al., 2013), while an S1R ligand significantly extended the lifespan in another ALS model (Mancuso et al., 2012). In addition, calcium-dependent proteases like calpains may be up regulated in the G93A mouse model (Gou-Fabregas et al., 2014; Stifanese et al., 2014) and may impair ion channel expression. Impairment in SK channel trafficking and expression on the membrane may trigger excitotoxic up regulation in N-type calcium currents that may increase MN degeneration (Pieri et al., 2013).

Thus, it is possible that a reduction in SK channel expression may be unintentional and a direct or indirect result of the SOD1 mutation, followed by compensation via alternative routes (calcium channel activation, Ih modulation, etc.). The largest channel cluster differences appear to occur later in disease progression, with changes in both SK2 and SK3 channels noted at the P90 and end-stage time points. This supports the idea that disease pathogenesis may be directly affecting SK channel reduction.

There is also evidence that suggests modulation of the VACHT-IR synapses that are found to be co-localized to SK2-IR and SK3-IR channel clusters in the SOD1-G93A model. Several groups have reported synaptic bouton size and density changes in the G93A motoneurons at various ages (Casas et al., 2013; Herron & Miles, 2012; Saxena et al., 2013). Data from the Elbasiouny laboratory also indicates that synaptic bouton size may be altered in mutant SOD1 motoneurons (Elbasiouny laboratory, unpublished data).
It would be important in the future to consider any potential changes in conjunction with the cluster changes reported here.

**SK channels mature over MN development**

The AHP is the time period after an action potential where the membrane potential of a motoneuron is hyperpolarized. Its length and amplitude are linked to the expression of SK channels (Deardorff et al., 2013). It is highly likely that AHP maturation may be tied to changes in SK channel expression. With developmental changes in both SK2 and SK3 cluster size as well as cluster intensity revealed through ANOVA analysis, it appears that channel clustering becomes more prominent as animals become older. This may suggest possible changes in SK activation happening over development. It is likely that SK channel density and clustering is closely affected by the maturation of their calcium sources (Carlin et al., 2000).

However, the number of cells expressing SK3 clusters appears to be higher early on and decreases over time. Estimates of the SK3+ populations of both WT and mutant SOD1 MNs at P90 and ES appear to resemble lower lumbar MN populations expressing SK3-IR in the rat and mouse (Deardorff et al., 2013). This is interesting given that neonatal motoneurons may have a longer mAHP duration and amplitude than adult motoneurons (Carrascal et al., 2005; Viana et al., 1994). The suggestion, then, is that there is an attenuation of the mAHP in some motoneurons with postnatal development, which could occur through many different possibilities (increased cell size or change in either SK or calcium channel properties).
Spinal motoneurons probably show a drastic increase in input conductance and cell size after P10, as supported by no demonstrated overall changes in input resistance and AHP parameters between P0-P3 and P8-P11 mice (Nakanishi & Whelan, 2010). However, the coefficients of variation for these parameters was significantly different at the older time point suggesting the start of major changes as well as possible differences between individual MNs. Thus, it is probable that a change in the mAHP is partly linked to increased cell size. A shortening of the mAHP is also generally linked and may be partly attributed to the development of the Ih current (Berger et al., 1996; Purvis & Butera, 2005). This current is hyperpolarization-activated and is thought to be active and depolarizing, leading to a shortening of the AHP. Data in this thesis suggests the involvement of a greater population of SK3-expressing, and putatively, long AHP, motoneurons early in murine life.

As mentioned earlier, it has been suggested that motoneurons in mice are relatively hypoexcitable as late as three weeks post birth (Chandran et al., 1991). There is also evidence that fast-twitch muscle fibers go through rapid growth between P21 and P42 in rat muscle (Miyata et al., 1996). Thus, decreased mAHP duration over neuronal maturation may be associated with changes in muscle fiber composition. This would help explain why it appears from the data that SK channels appear to not be fully mature in mice (reduced cluster intensity) at P30, an adult time point.

As SK3 channels have a longer activation and deactivation time constant than SK2 channels (Xia et al., 1998), this could implicate the disappearance of SK3 in the change in mAHP duration. The differences in MN discharge in the mouse in comparison to cats and rats have been attributed to the AHP (Manuel et al., 2009) and the presence of
SK3 in motoneurons has been implicated as a source of these changes (Deardorff et al., 2013). Widening the range of discharge during mouse MN development may allow proper muscle force gradation for producing motor output (Kernell et al., 1999). Thus, the disappearance of SK3 may play a role in increasing normal MN excitability during development.

**Dynamic Regulation of SK channels**

One has to note that the precise mechanisms of plasma membrane localization and trafficking regulation of SK channels are still not fully understood and may be important in determining the causes of the putative cluster changes detected in this study. It has been shown that calcium dependent cross-linking proteins like actinin 2 help influence membrane localization and expression of SK channels via cytoskeletal anchoring and control of recycling (Rafizadeh et al., 2014), and that changes in SK recycling result in changes in channel surface expression (Lu et al., 2009).

Lee et al demonstrated that calcium-independent interactions with calmodulin, which serves as the SK calcium sensor, are required for SK channel surface expression (Lee et al., 2003). It has been recently demonstrated that calmodulin interacts with other calcium-related membrane proteins to regulate cellular activity (Chou et al., 2015). SK channels also interact with protein kinase CK2 and protein phosphatase 2A, who are involved in modulating calcium gating and calcium-dependent modulation of SK channels (Allen et al., 2007; Bildl et al., 2004). These proteins all form a micro-complex that in sum or parts may be regulated by changes in neuronal activity and may influence SK channel activity.
It remains a possibility that changes in neuronal activity may influence signaling pathways that may lead to internalization of SK channels. Application of forskolin, a strong PKA activator, has been shown to internalize SK2 channels from neuronal and heterologous cell surfaces (Faber et al., 2008; Ren et al., 2006). In addition, deletion of various molecular domains of the SK3 protein including the calmodulin-binding domain has been shown to lead to retention of SK3 channels in the ER or cell body (Decimo et al., 2006; Roncarati et al., 2005).

**SK Channels and Neuronal Size**

From this thesis, larger MNs (based on soma size) on a whole appear to have larger SK clusters, in both SK2 and SK3 isoforms. This is interesting because the mAHP has been observed as being larger in amplitude and longer in duration in slow-twitch murine motoneurons (Deardorff et al., 2013). Differences in AHP amplitude between fast and slow motoneurons may be caused by differences in neuronal input resistance resulting from changes in somato-dendritic membrane size (Bakels & Kernell, 1993; Manuel et al., 2005; Zengel et al., 1985). Thus, it has been assumed that slow MNs (with larger input resistance) are smaller in size, compared to fast MNs. How then can it be possible that large MNs have a smaller AHP with larger SK clusters?

It is likely that SK cluster size and density measures *per unit membrane area* could approximate relative SK conductance in motoneurons. While cluster size generally appears to be correlated with soma size, it does not appear to be a strong correlation. This may indicate that per unit membrane area, smaller motoneurons actually have a larger amount of SK channel expression. This is interesting as calculated AHP conductance was
shown not to be different between slow and fast MNs, implying that slow motoneurons must be smaller in size therefore having a higher AHP conductance per unit membrane area (Bakels & Kernell, 1993). More information about cluster densities at all time points and in specific soma size ranges would be necessary to try to directly link AHP conductance with SK channel expression.

A small subset of large adult motoneurons was observed to express SK3-IR along with a restriction in soma size range (<1100 sq. microns). It is possible that a difference in SK isoform may affect and explain changes in AHP conductance. Indeed, it was shown previously that SK3-expressing MNs show physiological differences in AHP half-duration and amplitude (Deardorff et al., 2013). Here, the possibility remains that there may be populations of WT and mutant SOD1 MNs throughout the soma size range that express only one of these two isoforms (either SK2 or SK3). Unfortunately, as the antibodies used for SK2 and SK3 isoforms used in this thesis are made from the same species, it was not possible to do double-labeling studies. Given the availability of another SK2 or SK3 antibody, future studies should clarify these observations.

There is a possibility that another SK channel isoform, SK1, may play a role in differential expression in WT and mutant SOD1 MN populations. Little is known about this isoform and its presence in MNs, but it is thought to form heteromers with the SK2 isoform only (Monaghan et al., 2004). Thus, it is possible that its expression may be present only in SK3-IR negative cells (putative F-type). SK1 channels may be responsible for the SK currents regulating calcium PICs at the distal dendrites (Deardorff et al., 2013; Li & Bennett, 2007). However, it is conceivable that SK1 channels may also be somatic and may play a role in shaping total mAHP conductance.
Cluster combinations of these channels (SK1, SK2, and SK3) may underlie some of the variability that was observed in this thesis and in other studies. If and when a promising SK1 antibody is developed, it would be worthwhile to examine the complete distribution of the three isoforms in different alpha-MN types in WT and mutant SOD1 mice. These studies should be performed in conjunction with electrophysiological measurements to fully understand the potential role of each isoform on SK conductances in motoneurons.

Increases in neuronal soma size were detected in SK3-IR negative motoneurons, putatively the F-type motoneurons, in two early time points in the SOD1 mutant mice disease progression. Interestingly, a recent study noted an increase in soma size of mutant SOD1 motoneurons at P30 (Shoenfeld et al., 2014). Here, the data suggests that this increase in soma size is type-dependent and exists earlier in SOD1 motoneuron disease progression than thought before (Leroy et al., 2014). No change in soma size was detected at P90, and a decrease in soma size was detected in the end-stage mutant SOD1 motoneurons. This is likely due to the large amounts of cell death that is thought to be present in the last 30-50 days of mutant SOD1-G93A G1H mouse life (Fischer et al., 2004).

**Summary of Results**

The results from this thesis support the hypothesis that SK channel clustering is altered in lumbar α-MNs and provide molecular insight into possible changes in α-MN mAHP properties in mutant SOD1 pathogenesis. These changes may indicate a possible therapeutic target for ALS patients. In addition, the selective expression of SK3 appears
to be reduced early in mutant SOD1 disease pathogenesis. Furthermore, selective expression of SK3-IR appears to change during WT spinal cord maturation. These data offer insight into possible changes in neuronal maturation and neuromuscular activity in normal and disease conditions.

The results also support the hypothesis that soma size is increased in alpha-MNs over mutant SOD1 disease progression. However, increases in soma size appear to be present selectively in SK3- (disease-vulnerable MNs). Furthermore, there appears to be a reduction in MN soma size at late stages of disease pathogenesis, coinciding in time with large amounts of motoneuron death (Fischer et al., 2004). These changes confirm changes in soma size that appear to be present in mutant SOD1 mice. The specificity of soma size changes to vulnerable motoneuron populations (SK3- MNs) suggests that they may play an important role in disease pathogenesis.
VI. REFERENCES


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