Differential Loss of Spinal Interneurons in a Mouse Model of ALS

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Abstract—Amyotrophic lateral sclerosis (ALS) leads to a loss of specific motor neuron populations in the spinal cord and cortex. Emerging evidence suggests that interneurons may also be affected, but a detailed characterization of interneuron loss and its potential impacts on motor neuron loss and disease progression is lacking. To examine this issue, the fate of V1 inhibitory neurons during ALS was assessed in the ventral spinal cord using the SODG93A mouse model. The V1 population makes up ~30% of all ventral inhibitory neurons, ~50% of direct inhibitory synaptic contacts onto motor neuron cell bodies, and is thought to play a key role in modulating motor output, in part through recurrent and reciprocal inhibitory circuits. We find that approximately half of V1 inhibitory neurons are lost in SODG93A mice at late disease stages, but that this loss is delayed relative to the loss of motor neurons and V2a excitatory neurons. We further identify V1 subpopulations based on transcription factor expression that are differentially susceptible to degeneration in SODG93A mice. At an early disease stage, we show that V1 synaptic contacts with motor neuron cell bodies increase, suggesting an upregulation of inhibition before V1 neurons are lost in substantial numbers. These data support a model in which progressive changes in V1 synaptic contacts early in disease, and in select V1 subpopulations at later stages, represent a compensatory upregulation and then deleterious breakdown of specific interneuron circuits within the spinal cord.

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that is characterized by the adult-onset degeneration of motor neurons in the spinal cord and cortex. As disease progresses, patients suffer from increasingly severe motor deficits and eventual fatal paralysis (Hardiman et al., 2017). While motor neuron loss is a defining characteristic of ALS, the extent to which other components of motor circuitry are similarly affected or undergo compensatory changes remains unclear. A better understanding of the underlying cellular and circuit mechanisms of disease progression may be key in devising new treatments to prevent or slow ALS pathology.

Studies of the genetics underlying ALS have revealed that ALS-associated genes span many functional classes but together fail to reveal a common underlying mechanism of disease, suggesting that ALS is a syndrome with a complex etiology involving multiple pathways (Al-Chalabi and Hardiman, 2013). The first-identified ALS-associated gene, superoxide dismutase 1 (SOD1), has nonetheless been a focus of study, as mutations in this gene are associated with both familial (~20%) and sporadic (~5%) disease (Kaur et al., 2016). Transgenic mice expressing multiple copies of the SOD G93A mutant allele (hereafter referred to as SODG93A mice) phenocopy many of the neurological symptoms and cellular pathology seen in ALS patients, making this mouse line a valuable model to study ALS onset and progression (Lutz, 2018). This progression has been defined by clinical score (CS, Solomon et al., 2011): an early stage (CS1–2; postnatal days 85–110), when SODG93A mice first exhibit shaking and splaying of the limbs followed by deficits in stride and gait; a mid-stage (CS3–4; postnatal days 110–130), when
This progressive loss of motor function in ALS is accompanied by a prominent but selective loss of motor neurons, a feature that has historically defined the disease. In SOD\textsuperscript{G93A} mice, this loss becomes more severe with time, with a 30% reduction in motor neuron number by early and a 70% reduction by late stages of disease (Chiu et al., 1995). Even at pre-symptomatic stages, motor neurons exhibit changes in electrical properties and synaptic transmission, disrupted axon transport, and cellular stress and caspase activation (reviewed in van Zundert et al., 2012). The prominent loss of motor neurons in both human and mouse models of ALS (Kanning et al., 2010), however, is selective, with certain motor neurons subtypes showing a marked susceptibility and others not. Alpha motor neurons, which drive skeletal muscle contraction, are lost in high numbers over the course of disease, while gamma motor neurons, which innervate muscle spindles and modulate muscle sensitivity, do not decrease in number, even at late stages of disease (Kaplan et al., 2014; Lalancette-Hébert et al., 2016; Morisaki et al., 2016). Moreover, alpha motor neurons themselves exhibit differences in susceptibility to degeneration, with the fast-fatigue subtype the most susceptible, the fast-fatigue-resistant subtype less so, and the slow subtype the least susceptible (Frey et al., 2000; Fischer et al., 2004; Pun et al., 2006). The underlying cause of these differences in susceptibility is not known but could be due to differences in the intrinsic physiology or connectivity of motor neuron subtypes.

While motor neuron pathology is thought to make a large contribution to ALS disease onset, dysfunction of other spinal neurons is likely to contribute to disease progression. In human ALS patients, spinal interneurons, defined cytologically, are lost in significant numbers (Stephens et al., 2006), while in ALS mouse models, interneuron losses have begun to be mapped to defined subsets (van Zundert et al., 2012; Brownstone and Lancelin, 2018). In SOD\textsuperscript{G93A} mice, inhibitory neurons, identified broadly by the expression of the neurotransmitters, GlyT2 and Gad67, upregulate pathological markers of stress and downregulate neurotransmitter expression, (Hossaini et al., 2011). Renshaw cells, a small subpopulation of inhibitory neurons that participate in direct recurrent inhibition of motor neurons, first increase their synaptic contacts onto motor neurons in a proposed compensatory manner and then themselves decrease in number at very late disease stages (Wootz et al., 2013). Excitatory neurons also show a marked decrease in numbers in SOD\textsuperscript{G93A} mice, with loss of the V2a population matching both the extent and timing of motor neuron loss (Romero et al., 2017). Finally, synapses derived from the cholinergic V0c population are found first to be enlarged and then lost during disease progression (Chang and Martin, 2009; Pullen and Athanasiou, 2009; Herron and Miles, 2012), with functional inactivation of this subpopulation in ALS mice affecting motor performance (Landoni et al., 2019; Konsolaki et al., submitted). These lines of evidence increasingly point to a disruption of interneuron circuits in ALS progression, and raise the question of what the mechanistic relationship is between motor neuron loss, interneuron loss and disease stage.

To address this question, a more detailed understanding is needed of how the different classes of spinal interneurons are affected during ALS. Accordingly, we focused on the ventral V1 inhibitory class of neurons, which are derived from a common progenitor domain that gives rise to ~30% of all the ventral inhibitory neurons in the spinal cord, and comprise ~50% of the total inhibitory synaptic contacts onto motor neurons (Zhang et al., 2014). This V1 class includes the well-studied IA-inhibitory (~13% of total V1) and Renshaw cell (~9% of total V1) subpopulations (Alvarez et al., 2005), which coordinate antagonist muscle pairs and mediate recurrent inhibition, respectively (Gosgnach et al., 2017). In addition, V1 interneurons can be further parsed molecularly into distinct subpopulations based on combinatorial transcription factor expression (Bikoff et al., 2016; Gabitto et al., 2016; Sweeney et al., 2018). This recent analysis suggests that the V1 population at all spinal cord levels can be assigned to four groups, termed clades, with emerging evidence of clade-specific expression, connectivity and functional properties. Each clade can be further subdivided into multiple subtypes based on their unique expression of different combination of transcription factors, raising the possibility these represent diverse functional subtypes required for motor output at each spinal cord level (Francius et al., 2013; Bikoff et al., 2016; Gabitto et al., 2016; Sweeney et al., 2018). We reasoned that this diversity makes the V1 population an ideal class to examine whether interneuron subtypes are differentially susceptible to ALS progression. Conversely, since the significance of V1 genetic diversity has yet to be fully explored, such analysis of V1 subsets in the context of ALS provides an opportunity to test whether these represent different functional populations.

We assess the number of V1 inhibitory neurons in control and SOD\textsuperscript{G93A} mice during disease progression, using a genetic tracer. At late stages of disease, we find that 40% of V1 inhibitory neurons on average are lost in SOD\textsuperscript{G93A} mice, compared with an approximately 60% loss of motor neurons, 60% loss of V2a excitatory neurons, and limited or no loss of other ventral, NeuN\textsuperscript{+} neurons. This result supports a preferential loss of V1 and V2a compared with other V0, V2b and V3 ventral classes of interneurons. At early stages of disease, we find that V1 inhibitory neurons are less affected than motor and V2a excitatory neurons, demonstrating that V1 loss is a later feature of disease. We further find that V1 inhibitory neurons fall into ALS-susceptible or resistant subpopulations, analogous to the differential susceptibility of motor neuron subtypes. Finally, at early stages of disease before V1 inhibitory neurons are lost in large numbers, we show that V1 synaptic contacts increase on ventral motor neuron cell bodies undergoing stress and death. This increase is consistent with but exceeds that previously observed for Renshaw cells (Wootz et al., 2013). Together these results reveal a generalized early increase in V1 to motor neuron synaptic contacts, and a subsequent selective loss of V1 subpop-
ulations. Such alterations in V1 circuits may point to a broader role of interneurons in modulating the progress-
and severity of ALS.

**EXPERIMENTAL PROCEDURES**

**Animals**

All experiments and procedures were performed according to NIH guidelines and approved by the Institutional Animal Care and Use Committee of the Salk Institute for Biological Studies. The following previously published mouse strains were used in this study: B6S/J-Tg(SOD1*G93A)1Gur/J, SODG93A (Gurney et al., 1994), En1::Cre (Sapir et al., 2004), and B6.Cg-Gt(Rosa)26Sortm14(CAG-tdTomato)Hze/J (otherwise known as Ai14; Madisen et al., 2010). Geno-
typing was performed using ExTaq DNA Polymerase (Takara, RR001C) and manufacturer’s recommended PCR conditions with the following primers and annealing temperatures: SODG93A offspring with primers oligR13 and oligMR114 (Jackson Laboratory, Bar Harbor, ME, USA) at an annealing temperature of 60 °C. En1::Cre off-
spring with Cre 1 (5'-CCGGTGACGTGACGAAAA CAGGCTCTA-3') and Cre 2 (5'-CTTCAGGCGGC GATGTAGAGC-3') primers at an annealing temperature of 59 °C, and Ai14 offspring with a combination of Rosa 4 (5'-TCAATGCGGGGTTGCTGT-3'), 10 (5'-CTCCTGCT GCCTCTGCTTTCT-3'), and 11 (5'-CGAGGCCGATCA CAGGCAATA-3') primers at an annealing temperature of 66 °C. Age- and gender-matched control or SODG93A litters expressing En1::Cre and Ai14 were used for analysis. Animals were sacrificed at early (clinical score 1–2, CS1-2; abnormal spasm of the hindlimbs and tremors when lifted by the tail; average age = 110 ± 4 days) or late (clinical score 5, CS5; significant paralysis such that they are unable to right themselves after 30 seconds; average age = 124 ± 7 days) disease stages, in accor-
dance with standardized phenotyping criteria defined in Solomon et al., 2011. Control and experimental mice were maintained on a congeneric B6S/J background. Near equal numbers of female and male mice were analyzed for all experiments and were used using standard husbandry and housing conditions.

**Immunohistochemistry**

Mice were anesthetized and subsequently perfused with 1× PBS and then 4% paraformaldehyde. The spinal cord was then dissected out, post-fixed in 4% PFA for two hours at 4 °C, washed with 1× PBS, and immersed in 30% sucrose for at least 12 h, until equalized, before being frozen in Tissue-Tek OCT Compound (Sakura). Cryostat sections (25 μm) were washed with 1X PBS with 0.2% Triton (PBT), incubated with 10% donkey serum (Millipore) in PBT for one hour at room temperature, and then primary antibody in PBT plus 10% donkey serum overnight at 4 °C. Sections were then rinsed with PBT, incubated with secondary antibody in PBT plus 10% donkey serum for 30 min at room temperature, washed in PBT, incubated with DAPI, mounted in PVA/DABCO, and coverslipped for imaging. Images were obtained on either a LSM 710 or 780 microscope (Zeiss) at a 512 × 512 or greater resolution using a Plan-Apochromat 20×/0.8 M27 objective (visualization of neurons) or a LSM 880 Rear Port Laser Scanning Confocal with an Airyscan FAST module (visualization of synapses).

Primary antibodies used were as follows: goat anti-Choline Acetyl-Transferase (ChAT, 1:200, Millipore Sigma, #AB144P), guinea pig anti-Chx10 (Hayashi et al., 2019), rabbit anti-NeuN (1:1000, Millipore Sigma, #ABN78), rabbit anti-ATF3 (1:2000, Millipore Sigma, #HPA001562), rabbit anti-cleaved Caspase 3 (cCasp3, 1:150, Cell Signaling Technology, #9661), goat anti-
FoxP2 (1:1000, Santa Cruz Biotechnology N-16, sc-
21069), goat anti-Sp8 (1:500, Santa Cruz Biotechnology, C-18, sc-104661), rat anti-Pou6f2 (Jessell Lab, RRID: AB_2665427), rabbit anti-Calbindin D28K (1:200, Swant, #CB38), rabbit anti-Nr4a2/Nurr1 (1:500; Santa Cruz Biotechnology, M-196, sc-5568), and guinea pig anti-
VGAT (1:1000; Synaptic Systems, #131 004). Don-
key anti-goat, anti-guinea pig, anti-rabbit, and anti-rat fluorescent-conjugated secondary antibodies (1:500; Green: Alexa Fluor 488; Red: Cy3 or DyLight 549; and Far-red: Cy5, Alexa Fluor 647 or DyLight649) were obtained from Jackson ImmunoResearch and DAPI from Sigma (1:400; D9542).

**Quantification of spinal neuron number and spatial distribution**

To determine the number and distribution of neurons in the spinal cord, confocal images were imported into Imaris (Bitplane) and spots were assigned to the nucleus of each neuron either manually or using the “Spots” function followed by manual validation. Thresholds were set to exclude non-specific background immunoreactivity. For transcription factor analysis, variations in levels of expression were not taken into consideration, resulting in a determination of “expressed” or “not expressed.” Analysis of the spatial position of each cell body, or spot, was performed as previously described (Bikoff et al., 2016; Gabitto et al., 2016). In short, Cartesian coordinates for each neuron were determined in the transverse spinal cord plane with respect to the midpoint of the central canal, defined as position (0,0), and the lateral and ventral-most edge of the gray matter. The compiled data from all hemi-
sections analyzed was then morphed onto a standardized spinal cord plane. A determination of “expressed” or “not expressed.” Analysis of the spatial position of each cell body, or spot, was performed as previously described (Bikoff et al., 2016; Gabitto et al., 2016). In short, Cartesian coordinates for each neuron were determined in the transverse spinal cord plane with respect to the midpoint of the central canal, defined as position (0,0), and the lateral and ventral-most edge of the gray matter. The compiled data from all hemi-
sections analyzed was then morphed onto a standardized semi-section that reflected the lumbar spinal cord shape at postnatal day zero (distance from central canal to lateral edge was 650 μm and distance from central canal to ventral edge was 400 μm) or postnatal day 120 (distance from cen-
tral canal to lateral edge was 840 μm and distance from central canal to ventral edge was 570 μm) levels. Spatial contour analysis was performed as previously described (Bikoff et al., 2016). Related MATLAB codes are available at https://github.com/BPHO-Salk/DifferentialLoss_ALS.git.

**Analysis of cell body synaptic contacts**

The cell bodies of motor neurons and the neurites of V1 inhibitory neurons were labeled with ChAT
immunohistochemistry and En1::Cre-driven TdTomato expression, respectively. To determine the number of V1 synaptic contacts with a given motor neuron, a 1560 × 1560 pixel resolution image z-stack image was acquired with a 0.2 μm z-step via an AIRYscan LSM 880 confocal microscope with a Plan-Apochromat 63×/1.4 Oil DIC M27 lens (Zeiss). Seven consecutive sections at the level of the nucleus were analyzed and on the single, middle section, the number of synaptic contacts with the motor neuron cell body or proximal dendrites per μm of the neuron’s circumference were scored according to the following criteria: (1) a TdTomato-positive synaptic enlargement adjacent to the cell body or proximal dendrite that spanned at least 3 consecutive sections, and (2) the presence of a contiguous TdTomato-positive V1 neuronal process in one or more sections adjacent to the enlarged synaptic contact. In control experiments (see Fig. S5), VGAT antibody co-labeling was used to validate that such TdTomato-positive enlargements with a contiguous neurite were synaptic contacts. The cell body circumference of a given motor neuron was determined by tracing its perimeter using Imaris measurement tools.

Quantification and statistical analyses

All statistical analyses are detailed in figure legends and supplementary Table S1. For each littermate control and SOD mouse, parallel sections were collected every 25 μm over an identical rostro-caudal segment of the lumbar (L3–5), thoracic (T4–6) or brachial (C4–6) spinal cord. Individual sections were then imaged at varying but evenly spaced intervals within this segment—with the first image being the most rostral and the last image being the most caudal. For each animal, an average neuron number per section was then calculated from all sections imaged. For a given number of animal pairs (n = animals), these animal averages were then used for all subsequent statistical analyses. Normality was tested across animal pairs with either a Kolgomorov–Smirnov (large n) or a Shapiro–Wilk (small n) test as appropriate. For normally distributed data, significance of differences between animal means were compared with a two-tailed Student’s t or one-way ANOVA plus post-hoc Tukey (parametric). For all other non-normal data, a Mann–Whitney or Kruskal–Wallis plus Dunn’s (non-parametric) test was performed. All data are represented as the mean ± sem for a given number of animals, unless otherwise noted.

RESULTS

Spinal motor and interneuron loss at late stages of ALS

To examine differential neuron loss in the ventral spinal cord during ALS, we scored V1 inhibitory, V2a excitatory, motor and total neurons in SODG93A mice (Gurney et al., 1994) at late stages of disease (Figs. 1A and S1A). This late stage timepoint was defined by the inability of SODG93A mice to right themselves after thirty seconds, the well-established, phenotyping criteria of clinical score 5 (CS5, also referred to as late stage; average age = 124 ± 7 days; (Solomon et al., 2011). V1 inhibitory neurons were genetically marked by En1::Cre (Sapir et al., 2004) and the CAG.lsl.tdTomato reporter allele (Ai14; Madisen et al., 2010). Other neuronal populations were scored based on antibody reactivity for established markers of cell type identity: V2a excitatory neurons express Chx10 (Ericson et al., 1997; Crone et al., 2008; Hayashi et al., 2018), and motor neurons express ChAT. NeuN expression was used as a rough measure of total neurons, although restricted expression in some classes like motor neurons (see below) can be used to define subtypes.

We focused our analysis on the lumbar L3–L5 spinal cord and analyzed a representative set of spinal cord sections from SODG93A mice along with their age- and gender- matched littermate controls. As previously shown (Gurney et al., 1994; Chiu et al., 1995), we found that 58% of the lumbar motor neurons were lost at CS5 (Fig. 1B), with both those located in the lateral (61%) and medial (55%) halves of the spinal cord equally affected (Fig. S1B, C). Motor neurons in the lumbar spinal cord have been reported to vary in their susceptibility, with muscle fiber-innervating alpha motor neurons being the most susceptible, and muscle spindle-innervating gamma motor neurons being the least susceptible (Lalancette-Hébert et al., 2016). We similarly found that alpha motor neurons (ChATNeuN + with a large cell body perimeter) were markedly reduced in SODG93A mice at CS5, while gamma motor neurons (ChATNeuN + with a small cell body perimeter) were unaffected (Fig. S1D–F). In addition, and in agreement with Romer et al. (2017), we found that V2a excitatory interneurons were reduced by 57% in SODG93A compared with control mice (Fig. 1D). Based on the substantial losses of both motor and excitatory neurons, we explored whether inhibitory neurons might be similarly affected in SODG93A mice at this late disease stage.

Analysis of the number of V1 inhibitory neurons, indelibly marked by En1::Cre expression, revealed a 39% reduction in SODG93A compared to control mice (Fig. 1C). By contrast, no significant difference was detected in the same control and SODG93A mice in the total number of ventral NeuN + neurons, after excluding motor and V1 inhibitory neurons and because V2a excitatory neurons comprise a minor component of the total (Fig. 1E). In summary, we find that a significant fraction of V1 inhibitory neurons are lost at late stages of disease, and that this loss in combination with that of motor neurons and V2a excitatory neurons is greater than that of other neurons in the ventral spinal cord.

Spinal motor and interneuron loss at early stages of ALS

Previous studies have demonstrated that inhibitory neurons are unaffected at presymptomatic stages of disease (Hossaini et al., 2011), raising the question of when the V1 losses we observe occur. To explore the progression of subtype-specific neuronal loss in ALS, we examined SODG93A mice at a symptomatic but early
stage of disease onset, a clinical score of 1–2 (CS1-2, also referred to as early stage; average age = 110 ± 4 days). At this stage, mice display hyperflexion of the hindlimbs when suspended by the tail but no other movement deficits during the locomotor step cycle (Solomon et al., 2011). Previously, it has been found that only the most highly susceptible, alpha fast-fatigue motor neurons, which make up approximately one third of the total, are lost at this stage, while other motor neurons are still present (Lalancette-Hebert et al., 2016). V2a excitatory neurons similarly are less affected at this earlier disease stage in SODG93A mice, with only a 20% loss in V2a neuron number (Romer et al., 2017). Consistent with these previous results, in the lumbar spinal cord of CS1-2 SODG93A mice compared to control littermates (Fig. 2A–D), we found that the number of motor neurons (Fig. 2C) and V2a excitatory neurons (Fig. 2D) are decreased on average by 39% and 29%, respectively. In contrast, V1 inhibitory neurons were reduced by only 13% at this earlier disease stage (Fig. 2E). To examine the relationship between the differential loss of neuron types within a given animal, we scored V1 inhibitory, V2a excitatory, and motor neuron numbers in adjacent spinal cord sections from pairs of gender- and aged-matched control and SODG93A littermates. We then plotted these data at early (CS1-2) and late (CS5) stages to compare the proportional magnitude of loss of each neuronal population between animals and over disease progression (Fig. 2F–H and Table S1). Across individual animal pairs and disease stages, motor, V2a and V1 losses were well-correlated, demonstrating a potential relationship between loss of these interconnected neuron types. However, the extent of loss varied with motor and V2a loss occurring in a near one-to-one ratio (Fig. 2F) and consistently greater than V1 loss (Fig. 2G, H).

To examine further whether the pathology of V1 inhibitory neurons is less advanced than motor neurons at early stages, we labeled lumbar sections of CS1-2 SODG93A mice with antibodies against the cell stress marker, ATF3 (Fig. S2A–C) and the cell death marker, cleaved Caspase 3 (cCasp3; Fig. S2D–F). At late stages of disease, both ATF3 and cCasp3 are detected in motor and interneurons (Li et al., 2000; Pasinelli...
et al., 2000; Hossaini et al., 2011). However, at CS1–2, while motor neurons that express these markers were readily observed, very little expression was detected in V1 inhibitory neurons in the same section, providing additional support that motor neurons are more advanced in their degeneration than V1 inhibitory neurons at this stage. Together these data suggest that a subset of V1 inhibitory interneurons are lost in ALS but that V1 degeneration occurs after that of motor neurons and V2a excitatory neurons.

**Clade analysis of inhibitory V1 subtypes in ALS**

V1 inhibitory neurons can be subdivided by their physiologically and molecular properties, leading to the view that the V1 class is comprised of numerous transcriptionally, anatomically and electrophysiologically diverse subpopulations (Gosgnach et al., 2017). Since only a third of the total V1 population has degenerated in SOD93A mice even at late stages of disease, we examined whether V1 subpopulations were differentially affected. To address this issue, we first tested whether the transcription factors that subdivide V1 inhibitory neurons at an early postnatal stage also mark similar subpopulations in the adult spinal cord.

We focused our analysis on the four major subgroups of V1 inhibitory neurons, termed clades, which are defined by the mutually exclusive expression of the transcription factors FoxP2, Sp8, Pou6f2, and MafA; include greater than 50% of all V1 inhibitory neurons; and differ in their connectivity and electrophysiological properties (Bikoff et al., 2016; Gabitto et al., 2016). Antibodies against Foxp2, Sp8 and Pou6f2 labeled subsets of V1 inhibitory neurons in the adult (p120–140) (Fig. 3A). MafA staining, which primarily marks Renshaw cells at p0, was not detected in the adult, and thus Calbindin immunoreactivity coupled with ventral cell body position was instead used as a Renshaw cell marker (Benito-Gonzalez and Alvarez, 2012). Strikingly, the percentages of V1 inhibitory neurons (indicating clade size) labeled with these four markers were similar at adult stages (Fig. 3B–E) to those observed at p0 (Bikoff et al., 2016; Sweeney et al., 2018).

Furthermore, at both early postnatal and adult time points, these clade markers showed zero overlap (data not shown), together included more than 50% of all V1 inhibitory neurons, and individually each labeled V1 subpopulations with a spatially-distinct but temporally-conserved settling position (Fig. 3B–E). Such differences in position between clades but similarities between stages can be most readily observed in the dorsomedial, dorsolateral, and ventral positioning of the V1Sp8 (Fig. 3C), V1Pou6f2 (Fig. 3D), and V1Calbindin (Fig. 3E) subpopulations, respectively. These results indicate that transcription factor expression continues to track the V1 clades into adult stages, further supporting that the clade designation is likely to have functional significance for mature V1 interneurons.

Such persistence of transcription factor expression also provides a tool to examine the susceptibility of each clade to degeneration in SOD93A mice at late CS5 stages of disease. We found that the most dramatic reduction occurred in the V1FoxP2 clade (Fig. 3F, 51%), with a smaller but significant reduction in the V1Sp8 clade (Fig. 3G, 38%) and no significant change in the V1Pou6f2 (Fig. 3H) or V1Calbindin (Fig. 3I) clades. To address the possibility that such differences in susceptibility may in part result from differences in clade size, we performed power analyses. For all clades, we had sufficient power to detect 30% or greater changes in cell number, indicating that undetected changes in V1Pou6f2 or V1Calbindin clades are likely to be small or non-existent. These data indicate that V1 loss differs in magnitude between clades, suggesting that V1 inhibitory neurons, like motor neurons, fail into ALS-susceptible and ALS-resistant subpopulations.

**Combinatorial analysis of V1 subsets in ALS**

Within clades, V1 inhibitory neurons can be further classified into smaller subsets by the combinatorial expression of transcription factors (Bikoff et al., 2016; Gabitto et al., 2016; Sweeney et al., 2018). Like the clades, these smaller subsets may represent functionally distinct subpopulations, rendering them differentially susceptible to ALS. To begin to address this issue, we focused our analysis on the V1FoxP2 clade because it is the largest and the most affected, and surveyed the expression of transcription factors that subdivide it at early postnatal stages (Bikoff et al., 2016). We found that Nr4a2 expression was maintained until adult stages, and divided the FoxP2 clade into a Nr4a2-Off (V1FoxP2+Nr4a2+, 75% of the clade) and Nr4a2-On (V1FoxP2+Nr4a2+, 25% of the clade) subpopulation, similar to what has been observed at p0 (Fig. 3J, K). This similar pattern of Nr4a2 expression at postnatal and adult stages lends support to the functional importance of these transcriptional distinctions within a clade.

We further evaluated these clade-derived V1 subsets in ALS at the late CS5 stage of disease. We find in SOD93A mice that the V1FoxP2+Nr4a2− inhibitory neurons are reduced in number by 78% (Fig. 3N), while

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**Fig. 2.** Lumbar motor and interneuron loss at disease onset. (A, B) Hemi-sections of the ventral L3–L5 spinal cord of a control (CTL, left) and a CS1–2 SOD93A (SOD, right) mouse were stained for markers of either motor ([A]: ChAT +, green) or V2a excitatory ([B]: Chx10 +, green) and V1 inhibitory (En1.Tot +, red) neurons. Scale bar = 100 μm. (C–E) Ventral neuron loss in the lumbar spinal cord of CS1–2 SOD93A mice versus littermate controls: motor neurons ([C]: P < 0.0001, 40% decrease), V2a excitatory neurons ([D]: P = 0.004, 29% decrease), and V1 inhibitory neurons ([E]: P = 0.008, 13% decrease). Scatter dot plots represent average neuron number per 25 μm lumbar hemi-section as described in detail in the legend to Fig. 1. (F–H) Animal-by-animal comparisons of motor neuron, V1 inhibitory, and V2a excitatory neuron loss at lumbar levels in CS1–2 (green) and CS5 (red) SOD93A mice: Motor versus V2a excitatory neurons (P: slope = 1.04; R² = 0.55), motor versus V1 inhibitory neurons ([G]: slope = 0.67; R² = 0.35), and V2a excitatory versus V1 inhibitory neurons ([H]: slope = 0.62; R² = 0.70). Each dot represents the percent decrease in mean SOD vs. CTL neuron number for a single littermate pair (see Table S1 for raw data).
total $V_1^{\text{FoxP2}}$ (Fig. 3L) and $V_1^{\text{FoxP2}+\text{Nr4a2}^-}$ neurons (Fig. 3M) are only reduced by half. Thus, a smaller clade-derived subset, like the clades themselves, can vary in their susceptibility, further supporting a model in which specific V1 microcircuits may be differentially lost during disease progression. One implication of this
model is that the \( V1^{\text{FoxP2}} \) clade is known to contain la-inhibitory neurons (Benito-Gonzalez and Alvarez, 2012), suggesting that the dramatic losses we observe for specific FoxP2 subsets might reflect a potential disruption of reciprocal inhibitory circuits in ALS.

Spinal interneuron susceptibility in ALS along the rostro-caudal axis

Variation in motor and inhibitory neuron identity based on physiological and molecular properties is also observed along the rostro-caudal body axis. At limb levels, distinct motor neuron subsets project to flexor-extensor muscles to drive such movements as running and grasping, while at torso-levels, innervate hypaxial and back musculature to control breathing, posture, and the autonomic nervous system (reviewed in Dassen and Jessell, 2003). Similarly, molecularly-distinct V1 subpopulations exist at limb and torso levels with their function as of yet unexplored (Francius et al., 2013; Sweeney et al., 2019). To investigate whether such rostro-caudal differences in motor circuits might confer differential ALS susceptibility, we examined whether and to what extent motor and V1 inhibitory neurons were lost at brachial and thoracic levels of control and SOD\(^{G93A}\) mice at the late CS5 stages of disease.

In agreement with previous studies in SOD\(^{G93A}\) mice (Chiu et al., 1995), at late CS5 stages of disease, we found that the magnitude of motor neuron loss at different spinal levels varied significantly, with 58% lost at lumbar levels, 41% at brachial levels, and 26% at thoracic levels (Fig. 4A). The decreased susceptibility of thoracic motor neurons can largely be explained by the resistance of the autonomic subpopulation to degeneration (Fig. S3), a finding observed previously in both SOD\(^{G93A}\) mice (Chiu et al., 1995) and human ALS patients (Wetts and Vaughn, 1996). Once this motor neuron population is accounted for, somatic motor neurons are equally affected along the entire rostro-caudal axis, with \( \sim 50\% \) lost at all spinal cord levels (Figs. 4A, C and S3). In a manner similar to somatic motor neurons, V1 inhibitory neuron loss also occurred to an equal extent along the entire rostro-caudal axis, with an \( \sim 40\% \) reduction at all spinal cord levels (Fig. 4B, C). This result may indicate that V1 inhibitory neuron loss is more associated with somatic than with autonomic motor neuron loss.

Given the variation in motor output and circuit identity along the body axis, we evaluated whether V1 subtypes might differ in susceptibility based on rostro-caudal position by refining our analysis to the V1 clades. We found at all spinal cord levels that neurons in the \( V1^{\text{FoxP2}} \) clade were lost to the same extent (Fig. 4D), and those in the \( V1^{\text{Pou4f2}} \) and \( V1^{\text{Calbindin}} \) clades were largely unaffected (Fig. 4F, G). However, the \( V1^{\text{Sp8}} \) clade varied in susceptibility by level in SOD\(^{G93A}\) mice: at limb levels, \( V1^{\text{Sp8}} \) neurons decreased in number by \( \sim 35\% \), while at thoracic levels, they did not significantly differ in number (Fig. 4E). These findings support a functional relationship between motor and V1 subsets at different positions along the rostro-caudal axis, with such a relationship potentially underlying differences in ALS circuit susceptibility.

V1 inhibitory – motor neuron synaptic connectivity at early stages of ALS

The results above show that V1 inhibitory neurons are not lost in substantial numbers at early stages of disease, suggesting that an early loss of V1 inhibitory neurons is not an initial cause of motor neuron loss. However, even though V1 neurons are still present, their synaptic contacts onto motor neurons could be disrupted at these earlier stages and lead to motor neuron dysfunction. To examine this possibility, we evaluated the number of V1 synaptic contacts made directly onto the cell body of motor neurons, first in control and then SOD\(^{G93A}\) mice.

To provide a baseline for our analysis of SOD\(^{G93A}\) mice, we first quantified the total (V1) and Renshaw (\( V1^{\text{Calbindin}} \)) synaptic contacts onto motor neuron cell bodies in control mice. V1 synaptic contacts were scored at \( \sim p110 \) by counting the number of En1::Cre driven TdTomato\(^{+}/VGAT^{+} \) boutons on the cell bodies of ChAT\(^{-} \) motor neurons in the lumbar spinal cord (Fig. 5A, B). As shown previously (Alvarez et al., 2005), ChAT\(^{-} \) motor neuron cell bodies at lumbar levels had an average number of 0.07 synapses per micrometer. Nearly every TdTomato\(^{+} \) bouton was VGAT\(^{+} \) (Fig. S4A, B), leading us to score only TdTomato in subsequent experiments. We then further subdivided V1 synaptic contacts onto motor neuron cell bodies based on Calbindin immunoreactivity and dorsal-ventral position in the spinal cord. This enabled us to score the number of total (V1), Renshaw (\( V1^{\text{Calbindin}} \)), and non-Renshaw (\( V1^{\text{Non-Calbindin}} \)) contacts. As shown previously (Carr et al., 1998; Bikoff et al., 2016), we found that Renshaw cells make up only a small portion of total V1 synaptic contacts onto motor neuron cell bodies, and that these \( V1^{\text{Calbindin}} \) contacts are more numerous on ventral (20% of total V1 contacts) than dorsal motor neurons (5%) (Fig. 5B).

We then determined whether the number of V1 synaptic contacts onto motor neuron cell bodies varied in control and SOD\(^{G93A}\) mice. We focused our analysis on the early CS1-2 timepoint, when motor neurons have not yet been lost in dramatic numbers (Fig. 2) but have begun to express markers of stress (10% ATF3\(^{+} \)) and cell death (10% cCasp3\(^{+} \)) (Fig. S2). To eliminate variation due to cell-to-cell differences in disease state, we restricted our analysis to motor neurons that are actively stressed (Fig. S4C; ATF3-positive) or dying (Fig. S4D; cleaved Casp3-positive) in SOD\(^{G93A}\) mice. At these early stages of disease, these stressed or dying motor neurons are likely to be of an alpha fast-fatigue or fast-fatigue resistant subtype (Lalancette-Hébert et al., 2016), and thus, for comparison, we scored the corresponding large sized motor neurons in control animals. We found that the total V1 synaptic contacts onto the cell bodies of stressed or dying motor neurons were similar (dorsal and middle) or increased significantly (ventral) in SOD\(^{G93A}\) versus control mice (Fig. 5C). This increase in ventral V1 to motor neuron cell body contacts occurred for both Calbindin\(^{+} \) (Fig. 5D), consistent with previous reports (Wootz et al., 2013), and Calbindin\(^{-} \) V1 synaptic contacts (Fig. 5E), with Calbindin\(^{-} \) synaptic contacts...
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comprising the majority of motor neuron inhibitory input. Taken together, these data indicate that not only Renshaw cell but total V1 synaptic contacts onto motor neuron cell bodies increase on stressed and dying motor neurons in SOD G93A mice, arguing against a premature loss of V1 synapses.
DISCUSSION

Increasing evidence suggests that deficits in motor output in ALS result not only from the initial loss of motor neurons but also from more widespread alteration of modulatory circuits that control their firing (van Zundert et al., 2012; King et al., 2016; Brownstone and Lancelin, 2018). Such alterations have not only been observed for spinal motor circuits, as we demonstrate here for the V1 inhibitory neuron class, but also for cortical motor circuits, in which compensatory changes and/or degeneration of cortical pre-motor subtypes occur (Zhang et al., 2016; Clark et al., 2017). Like motor neuron loss, these interneuron losses appear to be subtype-specific with certain classes, and even subpopulations within these classes, most affected—as we demonstrate here for the V1 clades and a clade-derived subpopulation. This growing evidence of selective, circuit-level changes raises several fundamental questions about the mechanisms that initiate and propagate ALS pathology, including what the origin is of ALS susceptibility, and whether a functional relationship exists between ALS-susceptible motor and interneuron subtypes in both the normal and diseased state.

Selective loss of spinal interneurons

We show here that subsets of spinal interneurons are lost in the SOD\textsuperscript{G93A} mouse model of ALS at late stages of disease. Our data support the idea that like motor neurons, V1 and V2a interneurons have an increased susceptibility compared to other interneuron classes. Approximately half of V1 inhibitory and a quarter of V2a excitatory neurons are preserved, even in the most affected animals, suggesting the existence of resistant interneuron subpopulations, akin to the gamma subclass of motor neurons. Examination of V1 inhibitory subpopulations lends further support to this idea, as V1 clades and clade-derived subsets vary further in their susceptibility. Such differences in susceptibility may derive from differences in the downstream molecular profiles, connectivity or function of individual V1 subpopulations. We cannot completely exclude however that sampling biases due to variant subpopulation size or rostro-caudal distribution could also contribute to different levels of susceptibility. Moreover, clades that are unaffected in cell number could exhibit subtle alterations in health or synaptic connectivity, not detected by our current analysis. Additionally, because V1 clades comprise only half of total V1 inhibitory neurons, how the other half may differ in ALS susceptibility remains an open question. These observed and potential differences in V1 susceptibility mirror those of motor neurons, raising the question of how the two might be causally related during disease progression in ALS.

Potential mechanisms of selective interneuron susceptibility

Our results also raise the question of what underlies the differences we observe in spinal interneuron susceptibility, across all interneuron types and within the V1 subclass. It is noteworthy that the loss of V2a excitatory neurons we observe mirrors the timing and severity of motor neuron loss at both early and late stages of disease progression in SOD\textsuperscript{G93A} mice. The Chx10-expressing V2a excitatory neurons that we examine are known to make extensive direct synapses with motor neurons (Hayashi et al., 2018), and this direct connectivity could drive V2a loss either as a consequence of loss of their synaptic partners or through a trans-synaptic spreading of the disease, as has been previously proposed for the focal spread of human ALS (Ravits, 2014). Such a “direct-connectivity” model would predict that neurons require their postsynaptic partner for survival during ALS progression. A more detailed time course analysis of markers of degeneration in connected neurons, with higher spatial and temporal resolution and in combination with genetic or viral ablation of one of the two interconnected subpopulations, could shed light on the potential for degenerative, trans-synaptic spread.

In contrast to the matching of V2a and motor neuron degeneration, the loss of V1 inhibitory neurons is less than that of motor neurons during disease progression. At both early and late stages, the relative magnitude of motor neuron loss is greater than that of V1 loss on average and even within the same SOD\textsuperscript{G93A} mouse. One potential explanation of this incongruence is that V1 inhibitory neurons, unlike V2a excitatory neurons, may have variant patterns of connectivity with motor neurons, such that some are directly connected whereas others influence motor neuron output through indirect, multi-synaptic connections, and thus are less affected. For example, the most susceptible V1\textsuperscript{FoxP2} subpopulation includes la-inhibitory interneurons that regulate flexor–extensor coordination by directly connecting to motor neurons of antagonist muscle pairs (Benito-Gonzalez and Alvarez, 2012). Alternatively, V1 susceptibility may derive from connections to susceptible motor neurons. For example, the resistance of V1\textsuperscript{Pou6f2} and thoracic V1\textsuperscript{Sc8} inhibitory neurons may stem from the fact that these connect only with ALS-resistant motor neuron types.

This model, however, is at odds with the relative resistance of the Renshaw cell, a V1 subpopulation making direct synaptic contact on ALS susceptible motor neurons. Our data indicate that the loss of Renshaw cells is less pronounced than other V1 subpopulations in later stage ALS animals, although a previous report found a marked reduction in this population at even more severe disease stages (Wootz et al., 2013). The delayed susceptibility of the Renshaw cell subpopulation, despite their known direct connectivity to susceptible motor neurons, may reflect the specific properties of this V1 class. Renshaw cells are unique in their morphology, connectivity and intrinsic physiological properties (Alvarez and Fyffe, 2007), and are the only V1 inhibitory neuron that both receives input from and synapses onto motor neurons. Renshaw cells additionally express abundant calcium binding proteins, including Calbindin and Parvalbumin, and use both GABA and glycine inhibitory neurotransmitters, properties that have been correlated with the resistance of neuronal subtypes in...
the spinal cord, hindbrain and cortex (Alexianu et al., 1994; Appel et al., 2001; Lorenzo et al., 2006; Clark et al., 2017). Thus, the resistance of Renshaw cells may instead result from intrinsic resilience to disease-mediated degeneration.

This idea that intrinsic cellular properties might confer a greater susceptibility or resistance of some neuron classes compared to others also can be extended to the V1 clades, as they are by definition different in their transcriptional profiles. The consequences of these transcriptional signatures on cellular properties, while still largely unexplored, may include differences in neurotransmitter (GABA, Glycine, and GABA/Glycine), calcium binding proteins (Parvalbumin, Calbindin and Calretinin), or other intrinsic properties. On an anatomical level, V1 size and morphology could additionally vary between clades or differentially susceptible subpopulations and, on a physiological level, V1 inhibitory neurons at early postnatal stages exhibit markedly different active and passive membrane properties (Bikoff et al., 2016). Whether and how such intrinsic differences may contribute to selective susceptibility will become clearer as intrinsic properties are better defined for V1 clades under wildtype conditions. Our finding that the transcription factors that mark clades and clade-derived subtypes in early postnatal stages are conserved in their mutual exclusivity, cell body positioning, and relative numbers at adult stages lends some support to the idea that these subpopulations have different connectivity and functional properties that make them differentially susceptible to ALS.

V1 synaptic connectivity and ALS progression

Understanding how the changes we observe in spinal interneurons may influence motor neuron synaptic connectivity is crucial to unravel the role of motor circuits in ALS progression. Compensatory mechanisms to maintain circuit homeostasis have been proposed to explain the delay of any phenotypic symptoms of ALS until stages when greater than one third of motor neurons are lost. More specifically, such a compensatory mechanism has been suggested for Renshaw cells at the level of the synapse, wherein new Renshaw cell contacts onto motor neuron cell bodies appear at early stages of disease (Wootz et al., 2013). This finding suggests that inhibitory neurons at first may provide a protective effect by compensating for changes in motor neuron excitability and then, as they themselves degenerate, hasten disease progression and motor neuron loss—a model of circuit homeostasis (Brownstone and Lancelin, 2018).

Our analysis suggests that this homeostatic phenomenon is not restricted to Renshaw cells but may be a general feature of V1 inhibitory circuits, with total V1 synaptic contacts, both Calbindin-negative and Calbindin-positive, increasing on the cell bodies of stressed or dying motor neuron in SOD193A mice (Fig. 5). In fact, this increase in V1 synaptic number onto motor neuron cell bodies during disease progression is largely due to Calbindin-negative V1 inhibitory neurons, as they make up the bulk of direct inhibitory synapses in both a normal and diseased state. Several questions remain to be explored including whether this increase in cell body synaptic contacts occurs across the entire dendritic tree, whether it differs for each V1 clade, and how it may modulate motor neuron activity. Nonetheless, in a similar manner, an early increase in synaptic contacts has also been observed for the cholinergic V0c class (reviewed in Witts et al., 2014) and has yet to be explored for the excitatory V2a class, suggestive that modulation of synapse numbers occurs for other non-inhibitory neurons and raising the larger question of how the inhibitory-excitatory synaptic balance may be maintained or lost during ALS progression. Disruption of the inhibitory-excitatory synaptic balance at different disease stages and/or for different neuron types has the potential to contribute to the observed hypo or hyper-excitability phenotypes proposed to exacerbate ALS disease pathology (Van Den Bosch et al., 2006; de Martinez-Silva et al., 2018). A more detailed analysis of the inhibitory and excitatory circuits, and their relationship to motor neuron loss in ALS, is likely to be critical for understanding, and perhaps someday slowing, disease progression.

ACKNOWLEDGEMENTS

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REFERENCES


APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuroscience.2020.08.011.

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