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Voltage-gated calcium channels are abnormal in cultured spinal motoneurons in the G93A-SOD1 transgenic mouse model of ALS

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Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by progressive loss of motoneurons. Hyperexcitability and excitotoxicity have been implicated in the early pathogenesis of ALS. Studies addressing excitotoxic motoneuron death and intracellular Ca^{2+} overload have mostly focused on Ca^{2+} influx through AMPA glutamate receptors. However, intrinsic excitability of motoneurons through voltage-gated ion channels may also have a role in the neurodegeneration. In this study we examined the function and localization of voltage-gated Ca^{2+} channels in cultured spinal cord motoneurons from mice expressing a mutant form of human *superoxide dismutase-1* with a Gly93→Ala substitution (G93A-SOD1). Using whole-cell patch-clamp recordings, we showed that high voltage activated (HVA) Ca^{2+} currents are increased in G93A-SOD1 motoneurons, but low voltage activated Ca^{2+} currents are not affected. G93A-SOD1 motoneurons also have altered persistent Ca^{2+} current mediated by L-type Ca^{2+} channels. Quantitative single-cell RT-PCR revealed higher levels of Ca1a, Ca1b, Ca1c, and Ca1e subunit mRNA expression in G93A-SOD1 motoneurons, indicating that the increase of HVA Ca^{2+} currents may result from upregulation of Ca^{2+} channel mRNA expression in motoneurons. The localizations of the Ca1B N-type and Ca1D L-type Ca^{2+} channels in motoneurons were examined by immunocytochemistry and confocal microscopy. G93A-SOD1 motoneurons had increased Ca1B channels on the plasma membrane of soma and dendrites. Ca1D channels are similar on the plasma membrane of soma and lower on the plasma membrane of dendrites of G93A-SOD1 motoneurons. Our study demonstrates that voltage-gated Ca^{2+} channels have aberrant functions and localizations in ALS mouse motoneurons. The increased HVA Ca^{2+} currents and PC_{Ca} current could contribute to early pathogenesis of ALS.

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Keywords

Whole-cell patch-clamp; Quantitative single-cell RT-PCR; Calcium channel localization; Persistent Ca^{2+} current; G93A-SOD1 mice; high voltage activated Ca^{2+} currents

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset and fatal neurodegenerative disease characterized by progressive loss of motoneurons. About 90-95% of ALS is sporadic ALS with no known genetic component, and 5-10% is familial ALS (FALS) carrying mutations in genes such as the *SOD1* (*superoxide dismutase-1*), *TDP-43* (*TAR DNA-binding protein 43*), *FUS* (*fused in sarcoma*), and *C9ORF72* (*chromosome 9 open reading frame 72*) (Chen et al., 2013; Robberecht and Philips, 2013). Mutations in the *SOD1* gene occur in ~20% of FALS cases. *SOD1* gene mutations cause a toxic gain of function (Cleveland and Rothstein, 2001; Gurney et al., 1994). Transgenic mice expressing human *mutant SOD1* (*mSOD1*) genes develop fatal motoneuron disease resembling some aspects of ALS in humans (Gurney et al., 1994; Turner and Talbot, 2008). The precise mechanisms by which the mutant proteins lead to motoneuron degeneration in human and mouse ALS are still not understood. Multiple pathogenic processes have been implicated in *SOD1*-ALS pathogenesis, including oxidative stress, mitochondrial dysfunction, excitotoxicity, protein aggregation, impaired axonal transport, neuroinflammation, and dysregulated RNA metabolism (Ferraiuolo et al., 2011; Rothstein, 2009). Hyperexcitability and excitotoxicity may manifest early in ALS pathogenesis and are likely a node of convergence for multiple signaling cascades that leads to increased Ca^{2+} load of motoneurons (Van Den Bosch et al., 2006; van Zundert et al., 2012). Large α -motoneurons have low Ca^{2+} -buffering capacity and are particularly sensitive to intracellular Ca^{2+} challenges (von Lewinski and Keller, 2005). Studies addressing excitotoxic motoneuron death and intracellular Ca^{2+} overload have mostly focused on Ca^{2+} influx through α -amino-3-hydroxy-5-methylisoxazole propionic acid (AMPA) glutamate receptors (Grosskreutz et al., 2010; Heath and Shaw, 2002). However, the concept of exaggerated Ca^{2+} influx through glutamate receptors as a disease mechanism for ALS has not translated to effective new therapies for ALS (Musaro, 2012; Zoccolella et al., 2009). Ca^{2+} flux into motoneurons occurs also through activation of voltage-gated Ca^{2+} channels (VGCCs) following membrane depolarization, in addition to glutamate receptors. Intrinsic excitability of motoneurons is also a major contributor to excitotoxic vulnerability, and hyperexcitability is an electrical property abnormality that appears early in diseased motoneurons in ALS mouse models (Bories et al., 2007; Pambo-Pambo et al., 2009; Quinlan et al., 2011; van Zundert et al., 2012). Thus, there is uncertainty about whether other known routes of major Ca^{2+} entry to motoneurons contribute to disease mechanisms in ALS and thus provide new targets for therapy.

Several VGCCs have been characterized on the basis of pharmacological and physiological properties, and isoforms of the principal (pore-forming) α_1 subunit have been cloned (Catterall, 2000; Dolphin, 2009) (Table 1). VGCCs mediate inward Ca^{2+} currents that depolarize the cell membrane potential, modulate excitability, and provide intracellular Ca^{2+} signals that can activate gene transcription and neurotransmitter release (Ma et al., 2013).

Persistent Ca^{2+} current (PC_{Ca}) generated by Ca^{2+} channels is also very important in determining motoneuron excitability (Alaburda et al., 2002; Heckman et al., 2008). PC_{Ca} generates plateau potentials (Alaburda et al., 2002; Hounsgaard and Kiehn, 1989) and amplifies synaptic inputs to motoneurons (Bui et al., 2008; Powers et al., 2008). Many studies have shown that electrophysiological abnormalities are present embryonically in rodent models of ALS (Chang and Martin, 2011a; Kuo et al., 2004; Kuo et al., 2005; Pieri et al., 2009; Zona et al., 2006). These observations show directly that functional study of developing spinal cord slice and cellular models is relevant to identification of early mechanisms of ALS. In this study we tested the hypothesis that VGCCs have altered function and expression profiles in embryonic cultured motoneurons in an ALS mouse model. We used patch-clamp recordings to measure VGCCs, including PC_{Ca} , single-cell RT-PCR to quantify Ca^{2+} channel $\alpha 1$ subunit gene expressions, and immunocytochemistry to determine Ca^{2+} channel localizations in cultured spinal motoneurons from *mSOD1* transgenic mice.

Materials and methods

Transgenic mice

Transgenic mice expressing a human *mSOD1* gene encoding the glycine/alanine substitution at codon 93 (G93A) driven by the human SOD1 promoter (Gurney et al., 1994) and B6.Cg-Tg (Hlxb9-gfp)1Tmj/j transgenic mice expressing eGFP driven by the mouse Hlxb9 (Hb9) promoter (Wichterle et al., 2002) were originally obtained from the Jackson Laboratories (Bar Harbor, Maine) and then housed and bred in our animal facilities (Chang and Martin, 2009; Chang and Martin, 2011a). The G93A-SOD1 transgenic mice had a high copy number of mutant allele (~20 copies) and a rapid disease onset (Chang and Martin, 2009; Chang and Martin, 2011a). All experiments were approved by the Johns Hopkins University Institutional Animal Care and Use Committee (IACUC) and were conducted in accordance with the animal care guidelines outlined in the NIH Guide for the Care and Use of Laboratory Animals and the U.S. Public Health Service Policy on Use of Laboratory Animals. Every effort was made to minimize the number of animals used and their suffering.

Cell culture

To obtain embryos for spinal cord culture, G93A-SOD1 mice were mated with Hb9-eGFP mice. Breeder pairs were screened for the presence of the transgenes by PCR on tail DNA. On gestational day 13 (E13), female mice with potential double transgenic G93A-SOD1/Hb9-eGFP embryos and single transgenic Hb9-eGFP or G93A-SOD1 embryos were anesthetized with isoflurane and all viable embryos were harvested by caesarian section (Chang and Martin, 2011a). Hb9-eGFP expression in embryos was confirmed under a fluorescence microscope. Primary cultures were obtained from total spinal cords of male and female Hb9-eGFP⁺ embryos (Chang and Martin, 2011a). Dissociated spinal cord cultures were prepared and maintained as described previously (Chang and Martin, 2011a). Each spinal cord was cultured individually and each embryo was genotyped by PCR for the human *SOD1* gene after cell culture procedures (Chang and Martin, 2011a). All of the following experiments were conducted blinded of human *SOD1* genotype.

Electrophysiology

The Hb9-eGFP⁺ neurons were identified under fluorescent microscope and then recorded under differential interference contrast (DIC) optics. Whole-cell patch-clamp recordings were made with glass pipettes pulled on a P-97 electrode puller (Sutter Instruments, Novato, CA). For the recording of Ca²⁺ currents, the pipette was filled with an intracellular solution containing (in mM) 120 CsCl, 21 tetraethylammonium chloride (TEA-Cl), 0.5 4-aminopyridine (4-AP), 2 MgCl₂, 10 HEPES, 10 EGTA, 2 MgATP, 0.3 NaGTP, and 10 phosphocreatine, pH 7.25 adjusted with CsOH. Cells were superfused at a rate of 2 ml/min with an external bath solution containing the following (in mM): 120 NaCl, 30 TEA-Cl, 2 4-AP, 2.5 KCl, 10 HEPES, 10 D-glucose, 2 CaCl₂, 1 MgCl₂, and 0.5 μM tetrodotoxin (TTX), pH 7.3-7.4 adjusted with NaOH. For the recording of input resistance and action potentials (APs), the intracellular solution contained (in mM) 135 KCl, 2 MgCl₂, 10 HEPES, 10 EGTA, 2 MgATP, 0.3 NaGTP, 10 phosphocreatine, pH 7.25 adjusted with KOH, and the bath solution contained 150 NaCl, 2.5 KCl, 10 HEPES, 10 D-glucose, 2 CaCl₂, and 1 MgCl₂, pH 7.3-7.4 adjusted with NaOH. Recordings of spontaneous and miniature postsynaptic currents (PSCs) were performed by addition of different mixtures of synaptic transmission antagonists. For the recording of spontaneous excitatory postsynaptic currents (sEPSCs), glycine receptor antagonist (1 μM strychnine) and GABA receptor antagonist (5 μM bicuculline) were added to bath solution to block inhibitory synaptic transmission. For the recording of spontaneous inhibitory postsynaptic currents (sIPSCs), AMPA receptor antagonist [5 μM 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX)] and NMDA receptor antagonist [50 μM dl-2-amino-5-phosphonovalerate (APV)] were added to bath solution to block excitatory synaptic transmission. Miniature postsynaptic currents (mPSCs) were recorded by adding 0.5 μM TTX to bath solution. Experiments were performed at room temperature (22-24°C).

Currents from neurons were monitored with an Axopatch 200B amplifier (Molecular Devices, Palo Alto, CA), acquired through Digidata 1440A (Molecular Devices) onto a computer using pClamp 10 software (Molecular Devices). Data were analyzed using Clampfit 10.0 (Molecular Devices) and Origin 6.1 software (Origin lab, Northampton, MA). Linear leak and capacitive current were subtracted using a *p/4* protocol (Bezanilla and Armstrong, 1977). To assess the differences between motoneurons independently of cell size, the Ca²⁺ current recorded for each motoneuron was normalized with respect to the whole-cell surface area derived from measurements of the membrane capacitance. At least four independent cultures were used for the measurements of each parameter given in the results; and four to six motoneurons were recorded from each culture. After recording, individual motoneurons were randomly selected and genotyped by single-cell PCR for the human *SOD1* gene to confirm the existence of *mSOD1* gene (Chang and Martin, 2011a).

Activation curves of Ca²⁺ current were constructed with the conductance (*G*) calculated from the corresponding peak current using the equation

$$G = \frac{I}{V_t - V_r}$$

where V_t is the test potential, V_r is the reversal potential for the cell and I is the peak current amplitude (Carlin et al., 2000a). The conductance (G) for each cell was normalized to the maximum conductance (G_{max}) and plotted as a function of the test potential. Inactivation curves were constructed by normalizing the peak current amplitude (I) to the maximal amplitude of the Ca^{2+} current (I_{max}) and plotted as a function of the conditioning pulse potential. Values of the voltage dependency of activation or inactivation were fitted with the Boltzmann equation

$$\frac{G}{G_{max}} \text{ or } \frac{I}{I_{max}} = \frac{A_1 - A_2}{\left\{ 1 + \exp \left[\frac{V_t - V_{1/2}}{k} \right] + A_2 \right\}}$$

where A_1 is initial conductance or current, A_2 is final conductance or current, V_t is the test potential, $V_{1/2}$ is the voltage of half activation or inactivation, and k is the slope factor (Carlin et al., 2000a).

Motoneuron input resistance and AP properties were determined in current-clamp mode. The input resistance was measured by the slope of the linear portion of the current-voltage ($I-V$) curve obtained by injecting small pulses of currents. AP amplitude was calculated as the voltage difference between the AP threshold and peak of the overshoot. AP duration was the time interval between the voltage at one-half the height on the ascending and descending phase of the AP. The amplitude of the afterhyperpolarization (AHP) was measured as the voltage difference between firing threshold and voltage peak. Spontaneous and miniature PSCs were analyzed using Mini Analysis software (Synaptosoft, Decatur, GA). PSCs were detected with threshold amplitude of 5 pA (Mini Analysis) and verified by visual inspection. Cumulative fractional histograms of event intervals for spontaneous and miniature PSCs were computed and graphed using Mini Analysis. The statistical difference between these graphs was determined by the Kolmogorov-Smirnov test (Mini Analysis).

Ca^{2+} channel blockers (Table 1) ω -agatoxin TK (AgaTK), ω -conotoxin GVIA (CgTX) (Alomone Labs, Jerusalem, Israel) and $CdCl_2$ (Sigma) were prepared as stocks in external bath solution and kept frozen at $-20^\circ C$ until used. Stock solution of nifedipine (Alomone Labs) was dissolved in dimethyl sulfoxide and protected from the light. All drugs were made fresh from drug stock solutions and were bath applied.

PC_{Ca} measurement

Slow triangular voltage ramps (ramp speed 18 mV/s) were applied to Hb9-eGFP motoneurons to evoke the PC_{Ca}. Fig. 1, modified from (Lee and Heckman, 1998), illustrates the parameters that were retained for PC_{Ca} analysis. When the voltage was increased, the current initially increased proportionally, but later a deviation from this linearity leads to a zero slope region (Ion, Fig. 1). The current then decreased dramatically despite the continued increase in voltage, and formed a negative-slope region in the $I-V$ relation. This response is the N-shaped region (Schwindt and Crill, 1980) produced by PC_{Ca}. Another negative-slope region is also evident in the downward ramp. To obtain an estimation of the passive leak currents that sum with the PC_{Ca} to give the recorded current, a line fit was used

in the linear region and extrapolated to more positive voltages (estimated leak current; Fig. 1). The PC_{Ca} amplitude was estimated by subtracting the leak current from the recorded current (leak subtracted PC_{Ca}). The PC_{Ca} revealed after leak subtraction demonstrated a clear initial peak and sustained peak (Fig. 1, bottom trace). Following the terminology of Lee and Heckman (1998), the first zero slope point on the upward ramp in the recorded current was defined as the onset current (I_{on}) of the PC_{Ca} , and the corresponding voltage was defined as the onset voltage (V_{on}); the second zero slope point of the recorded current in the upward ramp was defined as the initial peak current (I_i) of the PC_{Ca} ; the first zero slope point on the downward ramp of the recorded current was defined as the sustained peak current (I_s) of the PC_{Ca} ; the second zero slope point on the downward ramp of the recorded current was defined as the offset current (I_{off}) of the PC_{Ca} and the corresponding voltage was defined as the offset voltage (V_{off} ; Fig. 1).

Quantitative single-cell reverse transcription-polymerase chain reaction (RT-PCR)

Single-cell RT and control reactions were performed as previously described (Chang and Martin, 2011a). Positive controls, using total RNA from mouse spinal cord, and negative controls, using water instead of RNA or without reverse transcriptase, were performed in parallel to the reactions for RNA isolated from single cells.

Following RT, the single-cell cDNAs for high voltage activated (HVA) Ca^{2+} channel $\alpha 1$ subunits (Ca1a-Ca1e; Table 1) and controls were preamplified using the TaqMan PreAmp Master Mix (Applied Biosystems, Grand Island, NY). A housekeeping gene β -actin was served as an endogenous control (Calvo et al., 2008). Ca1a-Ca1e primers/probes (TaqMan Gene Expression Assay) and β -actin TaqMan Endogenous Control were purchased from Applied Biosystems. The pooled assay mix was prepared by combining 6 of 20 $\times$ TaqMan Gene Expression Assays into a single tube and using 1 $\times$ TE buffer to dilute the pooled assays to a final concentration of 0.2 $\times$. The preamplification reaction mixture (40 μ l) consisted of 20 μ l of 2 $\times$ TaqMan PreAmp Master Mix, 10 μ l of 0.2 $\times$ pooled assay mix and 10 μ l of cDNA sample. The preamplification cycling conditions were 10 min at 95 $^{\circ}$ C following by 14 cycles of 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 4 min. The preamplified cDNAs were then diluted 1:20 with Tris-EDTA and subjected to single-plex quantitative PCR using TaqMan Gene Expression Master Mix (Applied Biosystems), as previously described (Chang and Martin, 2011a). PCR amplification was performed with 5 μ l of diluted preamplified DNA per reaction (in triplicate) for 50 cycles.

PCR efficiencies were calculated from the slope of the standard curve as previously described (Chang and Martin, 2011a). To confirm the linearity and parallelism of the preamplification step, non-preamplified standard curve cDNA was also amplified. Preamplification uniformity was checked by calculating the Ct values of each gene amplified from preamplified and non-preamplified cDNA, and determining the Ct between two Ct values ($Ct = C_{t, \text{preamp}} - C_{t, \text{cDNA}}$), as described by the manufacturer (Applied Biosystems TaqMan PreAmp Master Mix Kit Protocol, Appendix A: Checking Preamplification Uniformity). Ct values within ± 1.5 were considered uniformly preamplified and only uniformly amplified genes were selected for subsequent qPCR experiments.

Similarly, the single-cell cDNAs for acetylcholine-synthesizing enzyme choline acetyltransferase (ChAT), vesicular acetylcholine transporter (VACHT) (slc8a3), calcium/calmodulin-dependent protein kinase II α (CAMK2A) and control cDNA were also preamplified and then underwent PCR amplification as described above.

Results were calculated using the C_t method as previously described (Chang and Martin, 2011a) and statistical significance was determined using the Student's t test.

Immunocytochemistry

Spinal cord cultures grown on glass coverslips were fixed with 4% paraformaldehyde/PBS for 20 min, washed with PBS, permeabilized with 0.2% Triton X-100, blocked with 10% donkey serum, and then incubated in a cocktail of primary antibodies diluted in PBS containing 2% donkey serum and 0.05% Triton X-100 overnight at 4 °C (Chang and Martin, 2011a). The primary antibodies used in different combinations were: rabbit anti-L-type Ca^{2+} channel $\alpha 1D$ (Cav1.3) subunit (polyclonal, 1:100; Millipore, Billerica, MA); goat anti-N-type Ca^{2+} channel $\alpha 1B$ (Cav2.2) subunit (polyclonal, 1:20; Santa Cruz Biotechnology, Dallas, TX); mouse anti-synaptophysin (SYN) (monoclonal, 1:100; Dako Denmark A/S, Glostrup, Denmark), mouse anti-polysialic acid-NCAM (PSA-NCAM; monoclonal, 1:200, Millipore, Billerica, MA), mouse anti-SMI-32 (monoclonal, 1:1000; Covance, Princeton, NJ), and rabbit anti-manganese-containing SOD (MnSOD, SOD2; polyclonal, 1:400; Assay Designs, Ann Arbor, MI). Negative controls were assessed by first incubating the primary antibodies with control antigens for 1 h at room temperature and then incubating the mixture with the coverslips at 4°C overnight. After incubation in primary antibodies, the coverslips were washed four times with PBS, and then were incubated for 2 h at room temperature in a mixture of species-specific secondary antibodies (all raised in donkey) conjugated to Alexa Fluor 488, Alexa Fluor 594 and Alexa Fluor 647 (Invitrogen Corporation, Carlsbad, CA). Coverslips were washed again, and mounted using anti-fade mounting solution (Vectashield; Vector Laboratories, Burlingame, CA).

Image acquisition and quantification

Labeled cells were viewed and imaged using confocal microscopy as described previously (Chang and Martin, 2011a). All coverslips with spinal cord cultures were imaged under identical conditions and analyzed using identical parameters. Four to six embryonic cultures for each group were used for analysis. On each coverslip, four to six motoneurons were quantified.

Soma size and dendritic length of Hb9-eGFP motoneurons were measured by NIH Image J software. For each Hb9-eGFP motoneuron, z -series optical sections through the complete cell were collected and maximum intensity projection was derived from these sections. The soma and dendrites were delineated. Soma area, perimeter, feret diameter and dendritic length of the cell were calculated using Image J software. Number of branching points was manually counted. Hb9⁺ boutons were also manually counted and the density of Hb9⁺ boutons on soma was determined by dividing the number of boutons appearing to contact a motoneuron by the cell perimeter.

Measurements of Ca1B and Ca1D puncta numbers were performed using Image J software. The soma and dendrites of each Hb9-eGFP motoneuron were delineated and all immunoreactive puncta within the outlined cytoplasm and on plasma membranes were counted using Image J software. Threshold values of $0.05 \mu\text{m}^2$ and 50% of maximal intensity were chosen for defining puncta. For the determination of the immunoreactive puncta number on dendrites, proximal dendrites and distal secondary or tertiary dendrites that were 50-100 μm from the cell body and 0.5-1 μm in diameter were imaged and analyzed. Standardized puncta number (puncta density) was determined by dividing the number of puncta on the soma or dendrites by the cytoplasm area (puncta/ $100 \mu\text{m}^2$) or plasma membrane length (puncta/ $100 \mu\text{m}$). Similarly, measurements of large MnSOD-positive particles were also performed using Image J software. The soma of each motoneuron was delineated and all immunoreactive particles within the outlined soma were counted. Threshold values of $0.1 \mu\text{m}^2$ and 50% of maximal intensity were chosen for defining particles. Particle density was determined by dividing the number of particles in the soma by the soma area (particles/ $100 \mu\text{m}^2$).

Results are presented as mean $\pm$ SEM. Student's *t* test or two-way ANOVA followed by *post hoc* Tukey's test were used to assess statistical significance between control and G93A-SOD1 motoneurons. Differences were considered significant if $p < 0.05$.

Results

VGCC currents in control Hb9-eGFP motoneurons

Whole-cell patch-clamp recordings were performed on the soma of large Hb9-eGFP motoneurons cultured for 14-21 days. Motoneurons were identified by their eGFP expression, size, and characteristic morphology (Chang and Martin, 2011a). To examine the homogeneity of the large Hb9-eGFP cells (diameter $> 28 \mu\text{m}$) investigated, their somatic and dendritic morphologies were analyzed (Table 2). No significant differences were observed in soma area, perimeter, and Feret diameter between control and G93A-SOD1 large Hb9-eGFP motoneurons. Dendritic length and number of branching points were significantly reduced in G93A-SOD1 motoneurons compared to control motoneurons. We also compared the proportion of ChAT-expressing cells in control and G93A-SOD1 motoneurons. Due to the limitation of ChAT antibody staining of embryonic motoneuron cultures (Chang and Martin, 2011a), we analyzed ChAT and VACHT mRNA expression levels in Hb9-eGFP cells using quantitative single-cell real-time RT-PCR analysis. ChAT was detected in $\sim 73\%$ (11 of 15) control motoneurons and in $\sim 66\%$ (10 of 15) G93A-SOD1 motoneurons. ChAT mRNA expression in G93A-SOD1 motoneurons (the *Ct* value after normalizing to β -actin is 3.52 ± 0.58 , $n = 10$) was slightly lower compared to control motoneurons (*Ct* = 3.31 ± 0.45 , $n = 11$), but no significant difference was observed. VACHT was detected in all motoneurons. No significant change was observed in VACHT mRNA expression between control (the *Ct* value after normalizing to β -actin is 2.02 ± 0.21 , $n = 15$) and G93A-SOD1 motoneurons (*Ct* = 2.21 ± 0.29 , $n = 15$). Hb9-eGFP motoneurons were contacted by large Hb9⁺ boutons, resembling the C-boutons (Chang and Martin, 2009). We analyzed the densities of Hb9⁺ boutons and no significant differences were found between control ($4.6 \pm 0.6/100 \mu\text{m}$, $n = 38$) and G93A-SOD1 motoneurons ($4.1 \pm 0.7/100 \mu\text{m}$, $n = 39$). We concluded that the large

Hb9-eGFP neurons are uniform populations of cells within each group to allow for comparisons between control and G93A-SOD1 cultures.

VGCCs were isolated by occluding voltage-dependent Na^+ and K^+ currents with voltage-dependent Na^+ channel blocker TTX (0.5 μM) and K^+ channel blockers TEA⁺ (30 mM) and 4-AP (2 mM) in the external solution, and TEA⁺ (21 mM), 4-AP (0.5 mM), and Cs⁺ (120 mM) in the internal recording solution. Under this condition, whole-cell Ca^{2+} currents could be recorded in Hb9-eGFP motoneurons in response to 250 ms depolarizing steps from a holding potential of -90 mV to test potentials from -70 to 30 mV in 10 mV increments (Fig. 2a). The Ca^{2+} currents displayed two components. A fast transient component of current was first activated at a test potential of -60 mV, and a more sustained, slowly inactivating component appeared at more positive potentials (-30 mV; Fig. 2a). A change in the holding potential from -90 to -40 mV completely abolished the fast transient component, but the sustained, slowly inactivating component was apparent (Fig. 2b). A plot of the I - V relationship of the peak inward currents at two different holding potentials demonstrates two distinct Ca^{2+} currents with different activation ranges (Carlin et al., 2000a; Plant et al., 1998): low voltage activated (LVA, T-type) and HVA Ca^{2+} currents. The maximum current amplitude was at about -10 mV for both holding potentials. For voltage steps between -70 mV and -40 mV the I - V plot resulted only from LVA Ca^{2+} current (Fig. 2a, red triangles), because HVA Ca^{2+} currents were activated at about -30 mV (Fig. 2b). LVA Ca^{2+} current was fast and transient and showed activation at -60mV and maximum current at -40 mV (Fig. 2a, red triangles). For test potentials more depolarized than -40mV, LVA Ca^{2+} current could not be clearly isolated by simple subtraction, because capacitance artifacts elicited from a holding potential of -90mV differed in shape and amplitude from those elicited from a holding potential of -40mV. Therefore, we did not create I - V plots for pure LVA Ca^{2+} current over all test potentials from -50 mV to 30 mV. The HVA Ca^{2+} current showed a fast onset and did not fully inactivate during the 250 ms test potentials (Fig. 2). The mean maximum amplitudes for the LVA (-40 mV) and HVA (-10 mV) Ca^{2+} currents were 278 ± 12 pA ($n = 23$) and 785 ± 25 pA ($n = 17$), respectively. No significant difference was observed in Ca^{2+} currents in motoneurons cultured between 14 and 21 days *in vitro*.

HVA Ca^{2+} currents are increased in G93A-SOD1 motoneurons

We compared the Ca^{2+} currents originating in the somatic membrane in large Hb9-eGFP motoneurons from control and G93A-SOD1 mice (Fig. 3). HVA Ca^{2+} currents were evoked in all control and G93A-SOD1 motoneurons from a holding potential of -40 mV to test potentials from -50 mV to 10 mV (Fig. 3a). The peak Ca^{2+} current amplitude for each depolarizing step was normalized to the cell capacitance. The current density-voltage relationships are shown in Fig. 3b. HVA Ca^{2+} currents in both control and G93A-SOD1 motoneurons showed activation at -30 mV and maximum current at about -10 mV (Fig. 3b). G93A-SOD1 motoneurons ($n = 27$) had increased peak current densities of HVA Ca^{2+} currents compared to control motoneurons ($n = 23$; Fig. 3b). Significant differences were observed at -10, 0 and 10 mV. We did not attempt to measure the activation and inactivation time constants due to uncertainties in voltage control through the large dendritic tree of Hb9-eGFP motoneurons (Chang and Martin, 2011a), which is typical for spinal motoneurons (Carlin et al., 2000b). No significant difference was observed in HVA Ca^{2+} current densities

in medium-sized (diameter 10–28 μm) Hb9-eGFP⁺ neurons, presumably γ - or slow-type α -motoneurons (Chang and Martin, 2011b), between control ($n = 6$) and G93A-SOD1 cultures ($n = 6$) (Supplemental Fig. 1).

The voltage-dependent activation of the HVA Ca^{2+} current was analyzed in control and G93A-SOD1 motoneurons using 250 ms step pulses from a holding potential of -40 mV to test potentials from -50 to 0 mV in 10 mV increments (Fig. 3c). The data were fitted with a Boltzmann equation. The activation curve of G93A-SOD1 motoneurons shifted slightly to the right, but the half-activation voltage ($V_{1/2}$) and the slope factor (k) were not significantly different from control motoneurons. The steady-state inactivation of the HVA Ca^{2+} current was also analyzed. Currents were measured during a test potential of -10 mV preceded by 10 s conditioning pulses from -100 to -10 mV in 10 mV increments (Fig. 3d). Holding potential was set at -40 mV to inactivate the LVA Ca^{2+} current (Fig. 2b). Inactivation of the HVA Ca^{2+} current was incomplete even after the long conditioning pulses and a non-inactivating component remained (Fig. 3d). With a 10 s conditioning pulse, $15.2 \pm 2.8\%$ ($n = 6$) of the current was not inactivated in control motoneurons. For the current inactivated by the 10 s pulse, a Boltzmann distribution of the data showed similar $V_{1/2}$ and k in control and G93A-SOD1 motoneurons (Fig. 3d).

Pharmacology of the HVA Ca^{2+} currents in motoneurons

We characterized different subtypes of HVA Ca^{2+} channels by sequential application of a series of specific Ca^{2+} channel blockers in control and G93A-SOD1 motoneurons (Fig. 4a; Table 1). HVA Ca^{2+} currents were evoked every 60 s by a short (50 ms) depolarizing pulse to -10 mV from a holding potential of -40 mV. Bath perfusion of P/Q-type channel blocker AgaTK (100 nM) (Teramoto et al., 1995) slowly decreased part of the Ca^{2+} current in control motoneurons (Fig. 4a). Application of N-type channel blocker CgTX (3 μM) (Feldman et al., 1987) together with AgaTK in the bath solution produced a rapid further reduction of the current (Fig. 4a). Addition of L-type channel blocker nifedipine (20 μM) inhibited a small component of the current (Fig. 4a). A residual current remained, resistant to combined blockers application. Application of the non-selective Ca^{2+} channel blocker Cd^{2+} (100 μM) blocked nearly all of the remaining Ca^{2+} currents (Fig. 4a), indicating the presence of R-type Ca^{2+} currents (Randall and Tsien, 1995). The percentage of the current inhibition by these antagonists was calculated in control Hb9-eGFP motoneurons. AgaTK application blocked $\sim 15\%$ of the total currents in control Hb9-eGFP motoneurons. Subsequent application of CgTX reduced $\sim 34\%$ of the currents and nifedipine caused an additional $\sim 13\%$ reduction of the currents. Resistant current fractions were $\sim 40\%$ in control motoneurons. The inhibition of Cd^{2+} was $\sim 96\%$.

To identify the contribution of the specific subtype of Ca^{2+} current to the total enhanced HVA Ca^{2+} current in G93A-SOD1 motoneurons, the time course of peak Ca^{2+} current amplitudes during separate application of AgaTK (100 nM; Fig. 4b), CgTX (3 μM ; Fig. 4c), and nifedipine (20 μM ; Fig. 4d) in control and G93A-SOD1 motoneurons were examined. The results show that the N-type Ca^{2+} current contributes to the majority of the total increased Ca^{2+} current in G93A-SOD1 motoneurons. Fig. 4e summarizes the effects of the blockers as a mean percentage of total Ca^{2+} current in control and G93A-SOD1

motoneurons. The percentage of N-type Ca^{2+} current blocked by CgTX was significantly greater in G93A-SOD1 motoneurons than control motoneurons. No significant difference was observed in the percentage of P/Q- and L-type Ca^{2+} currents reduced by AgaTK and nifedipine between control and G93A-SOD1 motoneurons (Fig. 4e).

LVA Ca^{2+} currents are unaltered in G93A-SOD1 motoneurons

We compared LVA Ca^{2+} currents evoked by step pulses from a holding potential of -90 mV to test potentials from -70 mV to -40 mV in control and G93A-SOD1 motoneurons that showing LVA Ca^{2+} currents (Fig. 5a). The densities of LVA Ca^{2+} currents were slightly smaller in G93A-SOD1 motoneurons than in control motoneurons, but no significant differences were observed (mean current densities at -40 mV for control and G93A-SOD1 were 6.7 ± 1.7 pA/pF, $n = 17$ and 5.8 ± 1.3 pA/pF, $n = 15$, respectively).

We studied the voltage-dependent activation of the LVA Ca^{2+} currents by applying 150 ms step pulses to test potentials from -80 to -40 mV in 10 mV increments from a holding potential of -90 mV. The data were fitted with a Boltzmann equation (Fig. 5b). The LVA Ca^{2+} currents showed similar $V_{1/2}$ and k in control and G93A-SOD1 motoneurons. We measured the steady-state inactivation by applying conditioning pulses from -100 to -40 mV for 1 s in 10 mV increments followed by a test potential of -40 mV for 250 ms. Currents were reduced at conditioning potentials more positive than -80 mV, and little further reduction of the current was observed at conditioning potentials positive to -50 mV. $33.8 \pm 2.1\%$ ($n = 6$) of the current at -40 mV was resistant to inactivation and was probably HVA Ca^{2+} current (Fig. 2, 3). The inactivation curve for the transient component, after subtraction of the current that was not inactivated by the 1 s conditioning pulse, is shown in Fig. 5c. The $V_{1/2}$ and k of the inactivation curves were similar between control and G93A-SOD1 motoneurons.

PC_{Ca} is altered in G93A-SOD1 motoneurons

In the environment where Na^+ and K^+ channels were blocked, PC_{Ca} can be evoked when slow triangular voltage ramp commands (-120 to +60 to -120 mV over 20 s, ramp speed 18 mV/s) were applied to Hb9-eGFP motoneurons (Fig. 6a). The addition of nifedipine (20 μM) blocked the majority of the PC_{Ca} and the negative slope (Fig. 6a), indicating that the PC_{Ca} was mainly mediated by L-type Ca^{2+} channels. The data was then plotted as an I - V function with ascending and descending traces superimposed (Fig. 6b, right panel). Two types of I - V patterns were observed in Hb9-eGFP motoneurons. One type was overlapped with similar onset and offset voltages for upward and downward ramps (Fig. 6b, top right); the other type of I - V function showed a clockwise hysteresis (Fig. 6b, bottom right, arrows). In voltage step protocols, slow, delayed onset currents were seen in these motoneurons (Fig. 6c). Corresponding to its delayed onset, the current also deactivated slowly, evident by a large tail current following the depolarizing step (Fig. 6c, arrow). Both hysteresis (in ramp commands) and delayed onset (in step commands) Ca^{2+} currents are consistent with unclamped channels on the dendrites (Carlin et al., 2000b; Li and Bennett, 2003). The delay varied between individual motoneurons, perhaps due to the spatial distributions of dendritic L-type channels (Carlin et al., 2009; Grande et al., 2007). Thus, in some large Hb9-eGFP motoneurons, slow, delayed onset Ca^{2+} currents originating in the dendritic membranes were

detected (Fig. 6c). No delayed onset dendritic currents were seen in medium-sized Hb9-eGFP neurons (data not shown).

PC_{Ca} was observed in 83% ($n = 63$) control and 78% ($n = 56$) G93A-SOD1 large Hb9-eGFP motoneurons. 56% ($n = 52$) control motoneurons showed hysteresis and delayed onset Ca²⁺ currents. This proportion was reduced to 39% ($n = 44$) in G93A-SOD1 motoneurons. Table 3 summarizes the characteristics of PC_{Ca} in control and G93A-SOD1 motoneurons. G93A-SOD1 motoneurons had slightly more depolarized onset (V_{on}) and offset (V_{off}) voltages compared to control, but no significant difference was observed. Control motoneurons showed more hysteresis (ΔV) than G93A-SOD1 motoneurons, indicating more currents from further dendritic origin. The amplitude of the PC_{Ca} was quantified by measuring the initial (I_i) and sustained (I_s) peak amplitudes of the PC_{Ca} after subtraction of the leak current (see Materials and methods and Fig. 1 for detail). Both the initial peak (I_i) and the sustained peak (I_s) amplitudes and the current densities were significantly larger in G93A-SOD1 than in control motoneurons.

HVA Ca²⁺ channel mRNA expression is abnormal in G93A-SOD1 motoneurons

To examine molecular mechanisms underlying the HVA Ca²⁺ current abnormalities in G93A-SOD1 motoneurons, we used quantitative single-cell real-time RT-PCR to measure the five subtypes (Ca1a-Ca1e) of HVA Ca²⁺ channel $\alpha 1$ subunit (Table 1) mRNAs in individual G93A-SOD1 and control Hb9-eGFP motoneurons. A housekeeping gene β -actin was used as an endogenous control (Calvo et al., 2008). The standard curves for the Ca1a-Ca1e and β -actin showed approximately the same slope (data not shown), indicating similar amplification efficiency (Halford, 1999; Medhurst et al., 2000). Uniform preamplification was demonstrated by a Ct value between ± 1.5 when comparing the Ct values of each gene amplified from preamplified and non-preamplified cDNAs (data not shown).

Ca1a, Ca1b, Ca1c, and Ca1e mRNAs were detected in all Hb9-eGFP motoneurons. Ca1d was detected in 75% (18 of 24) control motoneurons and in 33% (9 of 27) G93A-SOD1 motoneurons. Fig. 7a shows amplification plots of Ca1a-Ca1e and β -actin transcripts obtained from two individual large Hb9-eGFP motoneurons (one from a control and one from a G93A-SOD1 culture) that are representative of the groups. Ct values (indicated by the bold line) were obtained for Ca1a-Ca1e and β -actin. Ct values for β -actin were similar in the two motoneurons (Fig. 7a), demonstrating β -actin was expressed in both control and G93A-SOD1 motoneurons in equal abundance and can be used to standardize the amount of RNA used in each RT-PCR reaction. Ca1a, Ca1b, Ca1c, and Ca1e transcript amounts in the G93A-SOD1 motoneuron were higher than that of the control motoneuron, indicated by lower Ct values in the G93A-SOD1 motoneuron (Fig. 7a). The data were normalized to β -actin levels and statistical analysis of the $Ct = Ct_{Ca1} - Ct_{\beta\text{-actin}}$ revealed a significant increase in mRNA expression of Ca1a, Ca1b, Ca1c, and Ca1e in G93A-SOD1 motoneurons compared to control motoneurons (Fig. 7b), showing that upregulation of *Ca1a*, *Ca1b*, *Ca1c*, and *Ca1e* gene expression coincides with the increase of HVA Ca²⁺ current in G93A-SOD1 motoneurons. Ca1d expression is low in both control and G93A-SOD1 motoneurons (Fig. 7a, b). No significant difference was observed in Ca1d expression between control and G93A-SOD1 motoneurons (Fig. 7b). To determine if the change in Ca²⁺ channel mRNA

level was selective for large motoneurons, medium-sized Hb9-eGFP⁺ neurons (Chang and Martin, 2011b) were also examined. No significant change was observed in Ca1a-Ca1e mRNA expression in medium-sized Hb9-eGFP⁺ cells between control and G93A-SOD1 cultures (Supplemental Fig. 2), indicating that the upregulated Ca²⁺ channel mRNA level is specific to large α -motoneurons.

Localizations of Ca1B and Ca1D Ca²⁺ channels are changed differentially in G93A-SOD1 motoneurons

To validate the mRNA expression data obtained from single-cell RT-PCR, we used immunocytochemistry to detect N-type and L-type Ca²⁺ channel $\alpha 1$ subunit proteins in cultured Hb9-eGFP motoneurons. We chose these two subtypes because N-type VGCC contributes to the majority of the total increased Ca²⁺ current in G93A-SOD1 motoneurons (Fig. 4) and L-type was shown to mediate PC_{Ca} (Carlin et al., 2000b). Antibodies that specifically recognize the $\alpha 1$ subunits of class B N-type (Ca1B; Cav2.2) and class D L-type (Ca1D; Cav1.3) Ca²⁺ channels (Table 1) were used to determine the localizations of these two subtypes in Hb9-eGFP motoneurons. Peptide blocking of antibodies confirmed the specificity of these antibodies to detect Ca²⁺ channels in motoneurons (data not shown).

Ca1B subunit was localized in cell bodies and dendrites of Hb9-eGFP motoneurons (Fig. 8a, 10). The Ca1B staining was not confined to the cell membrane but was also observed throughout the cytoplasm. In cell bodies, Ca1B staining was both diffuse and punctate in appearance, while in dendrites the labeling was mostly punctate and more prominent on the plasma membrane (Fig. 8a, 10). The immunoreactivity in dendrites was observed throughout the dendritic processes, and no apparent differences in the distribution of Ca1B were observed between proximal and distal dendrites of control motoneurons. Ca1B immunostaining was also observed in axon fibers and Hb9-eGFP axon terminals. Some punctate Ca1B immunoreactivities along the surface of motoneuron soma and dendrites were colocalized with presynaptic SYN (8a, purple, arrowheads), a ubiquitous synaptic vesicle marker (Navone et al., 1986), identifying these structures as presynaptic nerve terminal boutons. Colocalizations of Ca1B and SYN were found in axosomatic, axodendritic, and axoaxonic synapses (Fig. 8a, arrows and arrowheads). Large accumulations of Ca1B staining at synaptic structures were also observed. The punctate accumulations of Ca1B immunoreactivity might represent clusters of N-type Ca²⁺ channels in the presynaptic membrane of nerve terminals or in the postsynaptic membrane of the soma and dendritic shaft.

The Ca1D subunit immunoreactivity was found in cell bodies and dendrites of Hb9-eGFP motoneurons. A combination of diffuse and punctate staining was observed in the cell bodies and along the dendrites of motoneurons (Fig. 8b, 10). This pattern of staining suggests Ca1D localization in both synaptic and extra-synaptic sites. Ca1D immunoreactivity was more prominent in the cytoplasm of the soma and on the plasma membrane of the dendrites (Fig. 8b, 10), consistent with previous reports (Jiang et al., 1999; Westenbroek et al., 1998). This distribution can be due to active synthesis and trafficking of channels in constitutive secretory vesicles (Dolphin, 2009). Ca1D puncta on the plasma membrane would indicate the targeting of mature channels after synthesis. The staining in

dendrites was observed along the length of the dendrite, and no apparent differences in the distribution of Ca1D were observed between proximal and distal dendrites in control motoneurons. Ca1D immunoreactivity was also observed in nerve terminals (Fig. 8b). Different from the Ca1B immunostaining, the majority of SYN positive puncta were not associated with Ca1D puncta (Fig. 8b). A few Ca1D puncta located adjacent to SYN positive puncta, but the colocalization of the two proteins was not precisely co-aligned (Fig. 8b, arrowheads). SYN positive puncta were larger than the associated Ca1D puncta and appeared apposed rather than overlapping, indicating the postsynaptic localization of Ca1D. Arrows in Fig. 8b show colocalization of Ca1D and SYN in axoaxonic synapses. The axosomatic synapses formed by axon terminals from an Hb9-eGFP interneuron (Fig. 8b, dashed arrow) onto the Hb9-eGFP motoneuron are also shown. This finding suggests Ca1D L-type Ca^{2+} channels at presynaptic terminals may regulate neurotransmission at synapses. Ca1B and Ca1D immunoreactivities were also detected in other cells, including small Hb9-eGFP interneurons (Fig. 8b, dashed arrow). These results demonstrate that Ca1B and Ca1D are common Ca^{2+} channels used by different types of spinal cord neurons. To clarify the plasma membrane localization of Ca1B and Ca1D immunostaining, control neurons were co-stained with an antibody against the cell surface marker PSA-NCAM (Schlosshauer, 1989) (Fig. 9). Punctate Ca1B (Fig. 9, arrows) and Ca1D (Fig. 9, arrowheads) immunofluorescence were colocalized with PSA-NCAM on the plasma membrane of soma and dendrites.

We quantified Ca1B and Ca1D puncta densities in the cytoplasm and on the plasma membrane of soma and dendrites. Ca1B puncta on the plasma membrane represent both pre- and postsynaptic N-type Ca^{2+} channels, whereas Ca1D puncta on the plasma membrane mainly represent postsynaptic L-type Ca^{2+} channels. Ca1B puncta densities on the plasma membrane of soma and dendrites of G93A-SOD1 motoneurons ($n = 29$) were significantly higher than that of control motoneurons ($n = 32$) (Fig. 10c). Ca1D puncta densities were similar on the plasma membrane of soma between control and G93A-SOD1 motoneurons (Fig. 10c). Ca1D puncta densities on the plasma membrane of dendrites of G93A-SOD1 motoneurons ($n = 29$) were lower than that of control motoneurons ($n = 32$) (Fig. 10c). No significant differences were found in the Ca1B and Ca1D puncta densities within the cytoplasm of soma and dendrites between control ($n = 32$) and G93A-SOD1 motoneurons ($n = 29$) (Fig. 10c). No significant change was observed in the Ca1B and Ca1D puncta densities in medium-sized Hb9-eGFP⁺ neurons between control ($n = 9$) and G93A-SOD1 cultures ($n = 8$) (Supplemental Fig. 3).

G93A-SOD1 motoneurons in culture have enhanced excitability

Hyperexcitability is believed to be a hallmark during early pathogenesis of ALS (van Zundert et al., 2012). To examine if increased HVA Ca^{2+} and PC_{Ca} currents are accompanied by hyperexcitability, first we analyzed intrinsic excitability of control and G93A-SOD1 motoneurons. The passive membrane properties, the AP properties, and the pattern of AP firing were examined in motoneurons under current-clamp conditions. No significant differences were observed in resting membrane potential and input resistance between control and G93A-SOD1 motoneurons (Table 4). AP properties were similar for control and G93A-SOD1 motoneurons (Table 4). To examine the frequency-current (F-I)

relation, APs were recorded in response to increasing current injections (Fig 11a). G93A-SOD1 motoneurons fired at significantly higher frequency with current injection of 80, 100 and 120 pA (Fig 11b). Because VGCCs play important roles in neurotransmission (Catterall, 2011), next we examined if G93A-SOD1 motoneurons show increased synaptic transmission. The mean inter-event interval of sEPSCs and sIPSCs in G93A-SOD1 motoneurons was significantly shorter than in control motoneurons (Fig 12a). However, the mean inter-mEPSC and inter-mIPSC intervals were not altered in G93A-SOD1 motoneurons (Fig 12b), suggesting that increased event frequency was mainly due to increased activity-dependent vesicle release. Together, increased Ca^{2+} current is accompanied by both intrinsic hyperexcitability and enhanced synaptic transmissions in G93A-SOD1 motoneurons.

G93A-SOD1 motoneurons in culture have altered CAMKII expression and mitochondrial pathology

We also examined if downstream intracellular signaling pathways related to intracellular Ca^{2+} are abnormal in G93A-SOD1 motoneurons. CAMKII is a protein kinase that is regulated by the Ca^{2+} /calmodulin complex (Yamauchi, 2005). Using quantitative single-cell real-time RT-PCR, we measured the mRNA expression of CAMK2A in control and G93A-SOD1 motoneurons. CAMK2A mRNA expression in G93A-SOD1 motoneurons (the Ct value after normalizing to β -actin is 2.55 ± 0.25 , $n = 10$) was significantly increased compared to control motoneurons ($Ct = 3.08 \pm 0.37$, $n = 11$).

To identify other cytopathological changes that could be related to dysregulated Ca^{2+} channels in G93A-SOD1 motoneurons, we examined cytoplasmic vacuolization and/or mitochondrial swelling using an antibody against MnSOD (Chang and Martin, 2009). In control cultures, small, discrete, particulate-like or filament-like MnSOD-immunopositive mitochondria within cytoplasm were observed in SMI-32⁺ motoneurons (Fig 13a) and in other cells that were smaller neurons or putative astrocytes (Fig 13b, c). In G93A-SOD1 cultures, the MnSOD immunostaining in the cell bodies and proximal dendrites of some SMI-32⁺ motoneurons revealed prominent and large swollen mitochondria (Fig 13a). Mitochondrial swelling was relatively selective for G93A-SOD1 motoneurons in culture because it was not prominent in control cultures and was not apparent in other types of cells including SMI-32⁻ cells (Fig 13b) and astrocytes (Fig 13c).

We quantified large MnSOD-positive particles in the soma of control and G93A-SOD1 motoneurons. The number of MnSOD-positive particles in the soma of G93A-SOD1 motoneurons ($n = 22$) were significantly higher than that of control motoneurons ($n = 25$) (Fig. 13d). Similar mitochondrial pathology occurs in mSOD1 mouse motoneurons in spinal cord sections (Chang and Martin, 2009; Martin et al., 2007).

Discussion

The findings of this study relate to abnormalities in Ca^{2+} channel function, expression, and localization in spinal motoneurons in a mouse model of ALS. HVA Ca^{2+} currents are increased in G93A-SOD1 motoneurons, but LVA Ca^{2+} currents are not affected. G93A-SOD1 motoneurons also have altered PC_{Ca} mediated by L-type Ca^{2+} channels. The increase of HVA Ca^{2+} current could be due to upregulation of *Ca1a*, *Ca1b*, *Ca1c*, and *Ca1e* gene

expression and a higher density of Ca1B channels on the plasma membrane of G93A-SOD1 motoneurons. The increased HVA and PC_{Ca} currents could contribute to early pathogenesis of ALS.

Motoneurons in cell culture *versus* slice

We recorded VGCC currents and localized Ca²⁺ channels in motoneurons from mouse embryonic spinal cord cultures. Previous electrophysiological studies have identified VGCC currents in murine embryonic motoneurons (Baldelli et al., 1999; Hivert et al., 1995; Mynlieff and Beam, 1992a; Scamps et al., 1998; Viana et al., 1997). Cultured embryonic motoneurons and motoneurons from 7-day postnatal mice show qualitatively similar Ca²⁺ currents (Mynlieff and Beam, 1992b). We detected HVA Ca²⁺ current in all motoneurons. The Ca²⁺ currents in our motoneuron culture resemble physiologically and pharmacologically those in functionally mature mouse spinal motoneurons in acute slices (Carlin et al., 2000a). However, large motoneurons in slice preparations lack the full contribution of dendritic ion channels because the dendrites and axons are artifactually truncated by the vibratome cutting; moreover, rapid effects of drugs on motoneurons, including Ca²⁺ blockers, are difficult to assess because of diffusion barriers in the tissue slices, and motoneurons are difficult to keep alive in slices as acute slices are very temporary preparations rather than cultured and maintained preparations. Motoneurons in acute spinal cord slices of rat (Li and Bennett, 2003) and mouse (Jiang et al., 1999; Quinlan et al., 2011) have no or small PC_{Ca}. Motoneurons in our culture develop large cell bodies and elaborate dendrites (Chang and Martin, 2011a). The majority of motoneurons (83%) exhibited large amplitude PC_{Ca} and about half (56%) motoneurons had delayed dendritic currents. By immunocytochemistry we found robust Ca1B and Ca1D immunoreactivities in motoneurons at times when recordings were made. The cytoplasmic and membrane labeling of Ca1B and Ca1D was similar to that seen in mature rodent motoneurons *in vivo* (Jiang et al., 1999; Westenbroek et al., 1998). The pre- and post- synaptic localizations of Ca1B and Ca1D were confirmed by their associations with presynaptic terminals labeled with SYN. Taken together, these results suggest that our cultured motoneurons are well differentiated and mature with good dendritic structure that demonstrates PC_{Ca} and dendritic Ca²⁺ current. Despite the advantages of our model system, the cultured transgenic mouse neurons that we studied express mSOD1 at excessive non-physiological levels, so experiments using human familial ALS neurons are required (Boulting et al., 2011; Dimos et al., 2008).

VGCCs in wildtype motoneurons

We identified N-, P/Q-, L- and R-type channels in Hb9-eGFP motoneurons. Existing studies reported varying values for the proportion of currents mediated by VGCC subtypes (Carlin et al., 2000a; Mynlieff and Beam, 1992a; Mynlieff and Beam, 1994). This discrepancy could be due to variation in model preparations, alternative splicing of the $\alpha 1$ subunit (Bourinet et al., 1999; Gray et al., 2007), or variation in channel kinetics caused by differences in dendritic morphology (Carlin et al., 2000a).

We used single-cell RT-PCR to measure mRNA expression of Ca1a-Ca1e subunits in motoneurons. Ca1a and Ca1b subunits had the highest expression levels, whereas Ca1d subunit had much lower expression than the other four subtypes and was detected in only a

subset of motoneurons. Single-cell RT-PCR studies of motoneuron VGCC expression profiles are uncommon; our data are similar to the one existing study of rat facial motoneurons in slices (Plant et al., 1998). The discrepancy in the pharmacological results and the mRNA expression profiles could be explained by channel locations on motoneurons. For example, the presynaptic localization of P/Q-type channels may dominate over their postsynaptic localizations at the soma and dendrites (Westenbroek et al., 1998).

Using immunocytochemistry, we detected Ca1B and Ca1D subunit immunoreactivities in the soma and along the dendrites of motoneurons. The somal and dendritic Ca1D localizations in motoneurons of rodent spinal cord have been studied (Ballou et al., 2006; Carlin et al., 2000b; Jiang et al., 1999; Simon et al., 2003; Westenbroek et al., 1998; Zhang et al., 2006). However, there is little consensus on the precise location of Ca1D on motoneuron dendrites. We found Ca1D immunoreactivity decorating the length of dendrites with no apparent preference for proximal or distal domains in control motoneurons. Our results are consistent with studies of intracellular-filled cat motoneurons (Ballou et al., 2006) and with computational studies suggesting that L-type channels are located in discrete patches along the entire dendritic tree (Bui et al., 2006; Grande et al., 2007). By co-staining with SYN, we found that Ca1B channels localize pre- and post-synaptically, whereas Ca1D channels are mainly located post- and extra-synaptically. We detected the synaptic localizations of Ca1B and Ca1D on the soma and dendritic arbors of large motoneurons. These findings are consistent with previous reports (Westenbroek et al., 1998).

VGCCs in G93A-SOD1 motoneurons

We found that HVA Ca^{2+} currents are increased in G93A-SOD1 motoneurons. This abnormality could be caused by transcriptional upregulation of Ca^{2+} channel subunits. This hypothesis is supported by the single-cell RT-PCR finding of increased mRNA expression of *Ca1a*, *Ca1b*, *Ca1c*, and *Ca1e* genes in G93A-SOD1 motoneurons. The mRNA finding was substantiated at the protein level by increased plasma membrane Ca1B on G93A-SOD1 motoneurons as shown by immunofluorescence. Ca^{2+} channel trafficking and insertion into the plasma membrane needs to be assessed. A change in single-channel conductance or channel open time or probability of opening may contribute to the increase in Ca^{2+} currents as well.

The mechanisms by which mSOD1 alters Ca^{2+} currents and expression of Ca^{2+} channel subunits remain unclear. The altered VGCCs could be due to intrinsic changes in the properties of certain Ca^{2+} channels caused by mSOD1 directly or indirectly. mSOD1 pathophysiology is known to be associated with oxidative stress (Musaro, 2012). Oxidative stress could modulate the function of L-type VGCCs by altering their redox state (Li et al., 2007). Other possible mechanisms for VGCC abnormalities include mitochondrial functional and structural abnormalities (Fig. 13) and dendritic attrition in cultured G93A-SOD1 motoneurons. We observed reduced dendritic length and decreased number of branching points in G93A-SOD1 motoneurons. This dendritic attrition might lead to compensatory increase of VGCCs and synaptic transmissions (Martin et al., 2013).

The functional alteration in G93A-SOD1 motoneurons and the mechanism that cause this aberration appear to be selective for HVA Ca^{2+} currents because we did not find significant

changes in the LVA Ca^{2+} currents. An explanation for the differential changes on HVA and LVA Ca^{2+} channels in G93A-SOD1 motoneurons is not obvious. HVA Ca^{2+} currents and mRNA expression were not altered in medium-sized G93A-SOD1 Hb9-eGFP⁺ cells. The size of the soma and the dendritic structure of the medium-sized Hb9-eGFP⁺ motoneurons from which we recorded suggest that they could be γ - or slow-type α -motoneurons (Chang and Martin, 2011b). Our results indicate that modification of VGCCs early in the disease process of ALS is specific for large α -motoneurons, whereas the γ - and slow-type α -motoneurons are mostly spared. This specificity could be due to soma sizes and metabolic demands in different subtypes of motoneurons (Shaw and Eggett, 2000). Larger soma-sized motoneurons which innervate fast muscle fibers have high energy demands, high metabolic rates, and high levels of mitochondrial activities, thus are more vulnerable to neurodegeneration in ALS (Shaw and Eggett, 2000). The preferential vulnerability of large α -motoneurons and the relative resistance of γ - and slow-type motoneurons might also relate to their different firing patterns of APs (Burke, 1994). γ - and slow-type motoneurons have frequent, tonic firing that might be accompanied by more efficient ion and energy homeostasis mechanisms. Large α -motoneurons have phasic firing and are more susceptible to mitochondrial dysfunction and ion and energy homeostasis disruption (Shaw and Eggett, 2000; von Lewinski and Keller, 2005).

We analyzed the pharmacological contribution of various subtypes of Ca^{2+} currents to the total increase of HVA Ca^{2+} currents in G93A-SOD1 motoneurons using specific Ca^{2+} channel blockers. We found that the N-type Ca^{2+} current contributes to the majority of the total increased Ca^{2+} current in G93A-SOD1 motoneurons. This is confirmed by an increase in Ca1b mRNA expression, as well as an increase in Ca1B puncta on the plasma membrane of soma and dendrites. The reason for the discrepancy between the modest increase of N-type Ca^{2+} currents and the prominent increase of Ca1b mRNA and protein expression is not clear.

Our experiments demonstrate that cell physiology is abnormal long before mice develop symptoms because the motoneurons we studied were cultured (for 14-21 days) from embryonic spinal cords of control and mSOD1 mice. This conclusion is supported by evidence from acute neonatal mouse spinal cord slices indicating that the hyperexcitability and excitotoxicity in ALS are triggered much earlier, prior to the manifestation of symptoms (van Zundert et al., 2012). However, we cannot exclude the possibility that the mSOD1 alters the timing and sequencing of spinal cord maturational processes involving Ca^{2+} channels. Ca^{2+} channels contribute to many events during neuronal development, including cell migration (Komuro and Rakic, 1992), axonal growth (Gomez and Spitzer, 1999), and dendrite growth (Ryglewski et al., 2014). The developmental onset theory for ALS pathogenesis is refutable by the observation that G93A-SOD1 mice are born with locomotion mechanisms overtly intact and spinal cord development appears histologically normal by Nissl staining. Moreover, we did not observe significant difference in Ca^{2+} currents in wildtype and mSOD1 motoneurons cultured between 14 and 21 days *in vitro*. This suggests that the Ca^{2+} current is relatively constant in spinal motoneurons at this developmental stage during our recording period. However, mSOD1 mice do exhibit delays in acquiring sensory-motor skills early postnatally and show defects in NMDA- and serotonin-evoked rhythmic activity in spinal cord slices (Durand et al., 2006). Other neonatal

mouse spinal cord slice studies suggest that the acquisition of a variety of electrical properties of motoneurons is accelerated in G93A-SOD1 mice (Quinlan et al., 2011). Changes in voltage-gated ion channels in mSOD1 mice have been reported in previous studies. Altered voltage-dependent Na^+ channels were reported in cultured G93A-SOD1 spinal motoneurons (Zona et al., 2006) and mutant G93A transfected neuroblastoma cells (Zona et al., 1998). Increased expression of N-type Ca^{2+} channels was observed in cultured cortical neurons from G93A-SOD1 mice (Pieri et al., 2013).

PC_{Ca}

We recorded motoneuron PC_{Ca} and found that the PC_{Ca} was mediated mainly by nifedipine-sensitive L-type Ca^{2+} channels, consistent with previous reports on mouse and turtle motoneurons (Carlin et al., 2000b; Hounsgaard and Kiehn, 1989; Hounsgaard and Kiehn, 1993; Perrier and Hounsgaard, 2003). Moreover, we observed hysteresis (in ramp commands) and delayed onset (in step commands) Ca^{2+} currents that originate from unclamped distal dendrite of motoneurons (Carlin et al., 2000b). We found increased amplitude of PC_{Ca} but less hysteresis in G93A-SOD1 motoneurons. Factors influencing somatic current hysteresis are the dendritic location of Ca_{1D} channels relative to the soma, and the intrinsic activation/inactivation properties of these channels (Carlin et al., 2009; Muller and Lux, 1993). The decrease of the hysteresis current may suggest less contribution of distal dendritic currents in G93A-SOD1 motoneurons. This could be due to the dendritic attrition in G93A-SOD1 motoneurons. Another possibility is that L-type channels on distal dendrites of G93A-SOD1 motoneurons become abnormal functionally before channels on the soma or that the distal dendrites in general are degenerating. This interpretation is supported by the immunocytochemistry finding of decreased Ca_{1D} labeling on the membrane of distal dendrites of G93A-SOD1 motoneurons.

Motoneuron persistent inward currents (PICs) have been studied in mSOD1 rodents. Increased F-I gain was reported in G93A-SOD1 motoneurons in spinal cord cultures (Kuo et al., 2004; Kuo et al., 2005; Pieri et al., 2003) and in brainstem slices (van Zundert et al., 2008). Our observations with higher firing rates in G93A-SOD1 motoneurons are consistent with these findings. In contrast, reduced or unaltered F-I gain was observed in motoneurons from G85R-SOD1 brainstem-spinal cord preparations (Bories et al., 2007) and SOD1-G85R and G93A-SOD1 mouse spinal cord slices (Pambo-Pambo et al., 2009; Quinlan et al., 2011). The discrepancies between the intrinsic excitability changes in the different SOD1 transgenic mouse motoneurons could be related to the SOD1 variant, type of preparations, or type of recordings. The ionic mechanisms underlying altered F-I gain and PIC may involve the PC_{Na} and PC_{Ca}. Increased PC_{Na} has been demonstrated in mSOD1 mouse spinal (Kuo et al., 2005; Quinlan et al., 2011) and hypoglossal (van Zundert et al., 2008) motoneurons, and in cortical neurons (Pieri et al., 2009). However fewer studies focused on PC_{Ca}. Motoneurons in spinal cord slices from SOD1-G85R and G93A-SOD1 low-expresser mice showed increased Ca^{2+} component of total PIC, and fewer motoneurons with negative slope currents were identified (Pambo-Pambo et al., 2009). G93A-SOD1 motoneurons from spinal cord slices showed increased PC_{Ca}, but the amplitude of PC_{Ca} was relatively small in this preparation (Quinlan et al., 2011). However, in these two studies the K^+ currents, which could contaminate the PIC measurement, were not blocked when comparing the PC_{Ca}

between control and mSOD1 motoneurons. We specifically isolated PC_{Ca} by blocking Na^+ and K^+ channels. Our results are consistent with the previous studies. Moreover, these observations are consistent with a property of Riluzole, the only FDA-approved drug given for ALS, is inhibition of PC_{Ca} and PC_{Na} in murine spinal and hypoglossal motoneurons (Kuo et al., 2005; Lamanaskas and Nistri, 2008; Schuster et al., 2012) and that cultured motoneurons can be rescued from cell death by L-type Ca^{2+} channel antagonists (Arakawa et al., 2002; Roy et al., 1998; Tran et al., 2014).

Significance

Our results showed that increased HVA Ca^{2+} currents and altered PC_{Ca} in G93A-SOD1 motoneurons occur during early pathogenesis of ALS in mice. We found that altered Ca^{2+} currents coincide with motoneuron hyperexcitability. Other studies also reported altered motoneuron excitability in mouse models of ALS during the early stages of disease (Kuo et al., 2004; Kuo et al., 2005; Pambo-Pambo et al., 2009; Pieri et al., 2003; van Zundert et al., 2008; Zona et al., 2006). Increases in HVA Ca^{2+} currents and PC_{Ca} could cause increased Ca^{2+} influx following membrane depolarization. In addition, increases in PC_{Ca} may lead to an increased F-I gain and higher firing rates of motoneurons, as demonstrated in our study, thereby increase Ca^{2+} influx through activation of VGCC during the AP (ElBasiouny et al., 2010; Heckmann et al., 2005). The membrane depolarization induced by influx through PC_{Ca} will also cause sustained Ca^{2+} influx through other receptors/channels, such as NMDA- and AMPA-receptors, thereby directly contributing to excitotoxicity and mitochondrial dysfunction. Altered VGCCs may cause altered synaptic transmission. Increased VGCCs, particularly N-type and P/Q-type channels that are expressed at presynaptic terminals (Westenbroek et al., 1992; Westenbroek et al., 1995) may induce altered neurotransmitter release and consequently cause hyperexcitability/excitotoxicity through glutamate transmission. Increases in PC_{Ca} could amplify and prolong the synaptic inputs, thereby impacting output of the motoneuron by increasing its firing rates in response to synaptic modulation (ElBasiouny et al., 2010). We demonstrated enhanced synaptic transmissions in G93A-SOD1 motoneurons. Other studies also reported changes in excitatory and/or inhibitory synaptic activities in ALS mouse models (Chang and Martin, 2009; Chang and Martin, 2011a; Jiang et al., 2009; Quinlan et al., 2011; van Zundert et al., 2008). Whether these events result from a single signaling cascade or are independent or result from multiple pathways that interact in complex ways requires further study. Altered VGCCs could also affect numerous downstream intracellular signaling pathways in ALS pathology. Increased Ca^{2+} influx driven by VGCCs could increase protein kinase activities in ALS patients and mouse models (Hu et al., 2003a; Hu et al., 2003b; Krieger et al., 1996). We found increased CAMK2A mRNA expression in G93A-SOD1 motoneurons. The resulting increase in protein kinases in turn, may directly or indirectly influence the pathogenic process in ALS by modifying the phosphorylation of VGCCs, neurotransmitter receptors and structural proteins in a deteriorative feedback cycle.

Disruption of Ca^{2+} homeostasis has been implicated in ALS and plays an early and critical role in mSOD1 toxicity (Roy et al., 1998; Tradewell et al., 2011). Ca^{2+} influx through VGCCs and glutamate receptors, coupled with low cytosolic Ca^{2+} buffering, is a major factor in the preferential vulnerability of motoneurons to mSOD1 toxicity (Roy et al., 1998;

Shaw and Eggett, 2000; von Lewinski and Keller, 2005). As a consequence of mSOD1 toxicity, motoneurons failed to handle with Ca^{2+} overload, leading to mitochondrial dysfunction and eventually cell death (Tradewell et al., 2011). This hypothesis is consistent with our observation of mitochondrial swelling and cytoplasmic vacuolization in G93A-SOD1 motoneurons. Thus, maintaining Ca^{2+} homeostasis might be of therapeutic benefit in ALS. We have shown recently that increasing mitochondrial Ca^{2+} retention capacity with a mitochondrial permeability transition pore inhibitor has remarkable therapeutic effects in ALS mice (Martin et al., 2014). Other studies showed that limiting Ca^{2+} influx by manipulating glutamate receptors is protective in ALS mouse models (Tateno et al., 2004; Tortarolo et al., 2006; Van Damme et al., 2005; Van Damme et al., 2003). However, those strategies failed to translate to effective therapies for ALS patients (Zoccolella et al., 2009). Our findings of altered VGCCs in mSOD1 motoneurons suggest VGCCs as a target in ALS treatment. VGCC inhibitors have a long history of safe clinical use in cardiovascular and cerebrovascular diseases. Studies showed that inhibition of VGCCs partially prevents mSOD1 toxicity in cultured motoneurons (Roy et al., 1998; Tran et al., 2014).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ALS	Amyotrophic lateral sclerosis
SOD1	superoxide dismutase-1
AMPA	α-amino-3-hydroxy-5-methylisoxazole propionic acid
VGCC	voltage-gated Ca ²⁺ channel
PC_{Ca}	Persistent Ca ²⁺ current
mSOD1	mutant SOD1

TEA-Cl	tetraethylammonium chloride
4-AP	4-aminopyridine
TTX	tetrodotoxin
AP	action potential
PSC	postsynaptic current
sEPSC	spontaneous excitatory postsynaptic current
sIPSC	spontaneous inhibitory postsynaptic current
mEPSC	miniature excitatory postsynaptic current
mIPSC	miniature inhibitory postsynaptic current
CNQX	6-cyano-7-nitroquinoxaline-2, 3-dione
APV	2-amino-5-phosphonovalerate
I-V	current-voltage
AHP	afterhyperpolarization
AgaTK	ω -agatoxin TK
CgTX	ω -conotoxin GVIA
RT-PCR	reverse transcription-polymerase chain reaction
HVA	high voltage activated
LVA	low voltage activated
PIC	persistent inward current
F-I	frequency-current
PC_{Na}	persistent Na ⁺ current
ChAT	choline acetyltransferase
VAcHT	vesicular acetylcholine transporter
CAMK2A	calcium/calmodulin-dependent protein kinase II α
MnSOD	manganese-containing SOD
PSA-NCAM	polysialic acid-NCAM

Highlights

- We examined voltage-gated Ca^{2+} channels in cultured motoneurons in G93A-SOD1 mice.
- G93A-SOD1 motoneurons exhibit increased high voltage activated Ca^{2+} currents.
- G93A-SOD1 motoneurons also display altered persistent Ca^{2+} currents.
- G93A-SOD1 motoneurons have altered Ca^{2+} channel mRNA expression and immunoreactivity.
- Voltage-gated Ca^{2+} channels might contribute to ALS pathogenesis.

Fig. 1.

Measurement of persistent Ca^{2+} currents (PCCa) in Hb9-eGFP motoneurons. Whole-cell current response (middle trace) to a slow triangular voltage command (-120 to 60 to -120 mV over 20 s, ramp speed 18 mV/s) (top trace) is shown for an Hb9-eGFP motoneuron. The cell was held at -70 mV. According to the terminology of Lee and Heckman [38,40], I_{on} denotes the first zero slope point on upward ramp indicating the onset of PCCa ; V_{on} denotes the corresponding voltage to I_{on} ; I_i denotes the second zero slope point on upward ramp indicating the peak of PCCa ; I_s denotes the first zero slope point on downward ramp indicating the peak of PCCa ; I_{off} denotes the second zero slope point on downward ramp indicating the offset of PCCa ; V_{off} denotes the corresponding voltage to I_{off} . A line fit was used to extrapolate the leak current to more positive voltages (estimated leak current). The PCCa is represented by subtracting the leak current from the recorded current (leak subtracted PCCa).

Fig. 2.

Low voltage-activated (LVA) and high voltage-activated (HVA) Ca^{2+} currents in H9-eGFP motoneurons. **a**, Examples of traces of whole-cell Ca^{2+} currents (top) and their I - V plot (bottom) from an Hb9-eGFP motoneuron in response to 250 ms voltage steps from a holding potential of -90 mV to test potentials from -70 mV to 30 mV in 10 mV increments. Sample traces at -70, -50, -30, -10, and 10 mV are shown. The Ca^{2+} currents displayed two components: transient LVA and sustained HVA Ca^{2+} currents. Red triangles in I - V plot represent LVA component. **b**, Examples of traces of whole-cell Ca^{2+} currents (top) and their I - V plot (bottom) obtained from the same motoneuron as in **a** evoked by 250 ms voltage steps from a holding potential of -40 mV to test potentials from -50 mV to 30 mV. LVA Ca^{2+} currents were inactivated, but HVA Ca^{2+} currents were apparent at this holding potential.

Fig. 3.

HVA Ca^{2+} currents are increased in G93A-SOD1 motoneurons. **a**, Representative traces of HVA Ca^{2+} currents from control and G93A-SOD1 large Hb9 motoneurons, evoked by depolarizing step pulses (250 ms) from a holding potential of -40 mV to test potentials from -50 to 20 mV, step 20 mV, as shown in the *inset*. **b**, $I-V$ plots from control and G93A-SOD1 large Hb9 motoneurons. The peak current densities of HVA Ca^{2+} currents at different test potentials in G93A-SOD1 motoneurons ($n = 27$) are larger than in control motoneurons ($n = 23$). Significant differences were observed at -10, 0 and 10 mV. **c**, Voltage-dependent activation curves for HVA Ca^{2+} currents obtained from control and G93A-SOD1 large Hb9 motoneurons. Relative conductance (G/G_{max}) of HVA Ca^{2+} currents was plotted against test potentials. The activation curve of G93A-SOD1 motoneurons slightly shifted to the right, but no significant differences were detected between control ($n = 6$) and G93A-SOD1 motoneurons ($n = 6$). **d**, Voltage-dependent inactivation curves for HVA Ca^{2+} current obtained from control and G93A-SOD1 motoneurons. A test potential of -10 mV preceded by 10 s conditioning pulses from -100 to -10 mV in 10 mV increments were applied (*inset*). Normalized currents (I/I_{max}) were plotted against conditioning pulse potentials. Data were fitted with a Boltzmann curve. No significant differences were observed in the inactivation of the HVA Ca^{2+} currents between control ($n = 6$) and G93A-SOD1 motoneurons ($n = 6$).

** $p < 0.01$, * $p < 0.05$ between control and G93A-SOD1 motoneurons after two-way repeated measures ANOVA followed by *post hoc* Tukey's test.

Fig. 4.

Pharmacological isolation of HVA Ca²⁺ currents in control and G93A-SOD1 motoneurons. HVA Ca²⁺ currents were evoked every 60 s by 50 ms test pulses to -10 mV from a holding potential of -40 mV in control and G93A-SOD1 motoneurons. **a**, Top, Time course of peak current amplitude during sequential application of specific blockers of HVA Ca²⁺ channel subtypes in control (open circles) and G93A-SOD1 (filled circles) motoneurons. Sequential application of ω -agatoxin TK (AgaTK, 100 nM), ω -conotoxin GVIA (CgTX, 3 μ M), and nifedipine (20 μ M) reduced a portion of HVA Ca²⁺ currents but did not completely suppress HVA Ca²⁺ currents. Cd²⁺ (100 μ M) blocked nearly all the remaining currents. The horizontal bars indicate the duration of drug application. Bottom, Superimposed current traces obtained at different time points (labeled as 1-5) during drug application in control motoneurons. **b-d**, Time course of peak Ca²⁺ current amplitude during separate application of AgaTK (100 nM; **b**), CgTX (3 μ M; **c**), and nifedipine (20 μ M; **d**) in control (open circles) and G93A-SOD1 (filled circles) motoneurons. **e**, Comparison of the different subtypes of HVA Ca²⁺ currents in control and G93A-SOD1 motoneurons. Histogram shows the average percentage ($\pm$ SEM) of total Ca²⁺ currents blocked by AgaTK, CgTX, or nifedipine in control ($n = 5$) and G93A-SOD1 ($n = 5$) motoneurons, obtained in experiments similar to those shown in **b-d**. The percentage of N-type Ca²⁺ current blocked by CgTX was significantly greater ($*p < 0.05$, Student's t test) in G93A-SOD1 motoneurons than in control motoneurons.

Fig. 5.

LVA Ca^{2+} currents are not affected in G93A-SOD1 motoneurons. **a**, Representative current traces in control and G93A-SOD1 motoneurons, evoked by 150 ms step pulses to test potentials from -70 to -40 mV in 10 mV increments from a holding potential of -90 mV (inset). **b**, Voltage-dependent activation curves for LVA Ca^{2+} currents obtained from control and G93A-SOD1 motoneurons. Relative conductance (G/G_{max}) of LVA Ca^{2+} currents was plotted against test potential. **c**, Steady-state inactivation curves for LVA Ca^{2+} current obtained from control and G93A-SOD1 motoneurons. A test potential of -40 mV preceded by 1 s conditioning pulses from -100 to -40 mV in 10 mV increments were applied. Normalized current (I/I_{max}) was plotted against conditioning pulse potential. Data were fitted with a Boltzmann curve. No significant differences were observed in the activation and inactivation of the LVA Ca^{2+} currents between control ($n = 6$) and G93A-SOD1 ($n = 6$) motoneurons. Data represent the mean $\pm$ SEM (Student's t test).

Fig. 6. Persistent Ca^{2+} currents (PC_{Ca}) and dendritic currents in control Hb9-eGFP motoneurons. **a**, The current trace (bottom) with a negative-slope region (arrow) evoked by a slow triangular voltage command (top). See Fig. 1 for detailed explanations of trace components. Specific L-type Ca^{2+} channel blocker nifedipine ($20 \mu\text{M}$) blocked the majority of the PC_{Ca} and the negative slope. **b**, Two different types of I - V patterns for PC_{Ca} were seen in Hb9-eGFP motoneurons. Illustrative current response plotted as function of voltage applied (I - V plot, right), derived from the recorded current (left). Note the clockwise hysteresis loop (lower right trace, arrows) on downward ramp compared with upward ramp. The size of the hysteresis was quantified as the difference between the voltage at the onset of the PC_{Ca} and the offset of the PC_{Ca} ($V = V_{\text{on}} - V_{\text{off}}$). **c**, Currents recorded from the above motoneuron when a series of voltage steps of 150 ms duration from -50 to -10 mV in 10 mV increments were applied. Note the slow, delayed onset and offset (tail current; arrow) of PC_{Ca} originating in the dendritic membranes.

Fig. 7.

HVA Ca^{2+} channel $\alpha 1$ subunit mRNA expression is altered in G93A-SOD1 motoneurons. **a**, Representative real-time PCR amplification plots of Ca1a (black circles), Ca1b (red crosses), Ca1c (orange triangles), Ca1d (purple squares), Ca1e (blue diamonds) and β -actin (green stars) mRNA transcripts in a control and a G93A-SOD1 motoneurons. *Ct* for each transcript is shown as the intersection of the bold line with the RFU (relative fluorescence units) plot. **b**, Quantitative real-time RT-PCR analysis of *Ca1a-Ca1e* gene expression levels in control and G93A-SOD1 motoneurons. Expression levels of Ca1a-Ca1e mRNA were calculated using the *Ct* method. Except for the *Ca1d*, all other genes tested had higher mRNA expression levels in G93A-SOD1 motoneuron than in control motoneurons. Data are mean $\pm$ SEM (** $p < 0.01$, Student's *t* test). $n = 24$ for control and $n = 27$ for G93A-SOD1.

Fig. 8.

Low and high magnification of confocal microscope images show the localization of Ca1B N-type (**a**) and Ca1D L-type (**b**) Ca^{2+} channels co-stained with synaptophysin (SYN) in control Hb9-eGFP motoneurons. Some punctate Ca1B immunofluorescence on soma and dendrites were colocalized with SYN (**a**, arrowheads). Ca1B immunofluorescence was also observed in axons and axoaxonic synapses (**a**, arrows). The majority of SYN-positive puncta did not coincide with Ca1D puncta but were in some instances seen in apposition (**b**, arrowheads). Arrows in **b** show colocalization of Ca1D and SYN in axoaxonic synapses. Axosomatic synapses formed by axon boutons from an Hb9-eGFP interneuron (**b**, dashed arrow) onto the Hb9-eGFP motoneuron are also shown. Scale bars = 20 μm for low magnification images and 5 μm for high magnification images.

Fig. 9.

Representative confocal microscope images show the localization of Ca1B N-type and Ca1D L-type Ca^{2+} channels co-stained with cell surface marker PSA-NCAM in control neurons. Punctate Ca1B (arrows) and Ca1D (arrowheads) immunofluorescence colocalized with PSA-NCAM immunostaining on the plasma membrane of soma and dendrites. *, nucleus. Scale bars = 5 μm .

Fig. 10.

Ca1B and Ca1D Ca^{2+} channel subunit localizations are altered in G93A-SOD1 motoneurons. **a-b**, Representative confocal images showing Ca1B and Ca1D immunoreactivities in soma (**a**) and dendrites (**b**) of control and G93A-SOD1 Hb9-eGFP motoneurons. The motoneurons shown receive elaborate axosomatic innervations from Hb9-eGFP axons. **c**, Quantitative analysis of Ca1B and Ca1D immunoreactive puncta densities on the soma and dendrites of control ($n = 32$) and G93A-SOD1 ($n = 29$) Hb9-eGFP motoneurons. Histograms showing the distribution of Ca1B and Ca1D puncta in the cytoplasm and on the plasma membrane relative to somatic and dendritic area or length. Data represent the mean $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$, Student's t test). Scale bar = 10 μm .

Fig. 11.

Intrinsic hyperexcitability in G93A-SOD1 motoneurons. **a**, Representative traces of membrane potentials evoked by current injections (bottom) in control (top) and G93A-SOD1 (middle) motoneurons under current-clamp conditions. **b**, Mean action potential (AP) firing frequency plotted against injected currents (F-I plot) in control ($n = 12$) and G93A-SOD1 motoneurons ($n = 11$). The AP firing frequency was significantly increased in G93A-SOD1 motoneurons at 80, 100 and 120 pA, indicating the hyperexcitability of G93A-SOD1 motoneurons. Data represent the mean $\pm$ SEM. ** $p < 0.01$, * $p < 0.05$ between control and G93A-SOD1 motoneurons after one-way repeated measures ANOVA followed by *post hoc* Tukey's test.

Fig. 12.

Synaptic transmission is altered in G93A-SOD1 motoneurons. **a**, Representative traces of spontaneous excitatory postsynaptic currents (sEPSCs) and spontaneous inhibitory postsynaptic currents (sIPSCs), and the cumulative histograms of event intervals for sEPSCs and sIPSCs were shown for a given recording in control and G93A-SOD1 motoneurons. The frequency of sEPSCs and sIPSCs was significantly increased in G93A-SOD1 motoneurons. **b**, Representative traces of miniature excitatory postsynaptic currents (mEPSCs) and miniature inhibitory postsynaptic currents (mIPSCs), and the cumulative histograms of event intervals for mEPSCs and mIPSCs were shown for a given recording in control and G93A-SOD1 motoneurons. No significant difference was observed in the frequency of mEPSCs and mIPSCs between control and G93A-SOD1 motoneurons. Holding potential, -70 mV. The statistical difference between these graphs was determined by the Kolmogorov-Smirnov test (Mini Analysis).

Fig. 13.

Representative confocal images showing MnSOD immunoreactivities (red) in control and G93A-SOD1 cultures. Large swollen MnSOD-positive mitochondria were observed in SMI-32⁺ (green) motoneurons in G93A-SOD1 cultures (**a**). No obvious MnSOD-positive mitochondrial swelling was observed in SMI-32⁻ cells (**b**) and astrocytes (**c**) in G93A-SOD1 cultures. **d**, Quantitative analysis of large MnSOD-positive particle densities in the soma of control ($n = 25$) and G93A-SOD1 ($n = 22$) motoneurons. Histograms showing the number of MnSOD-positive particles in the soma relative to somatic area. Data represent the mean $\pm$ SEM (** $p < 0.01$, Student's t test). *, nucleus. Scale bar = 10 μ m.

Table 1

Nomenclatures of voltage-gated Ca^{2+} channels.

Current type	Specific blocker	Activation voltage	$\alpha 1$ subunit gene name
L-type	Dihydropyridine	HVA	Cav1.1 (CACNA1S/Ca1s)
			Cav1.2 (CACNA1C/Ca1c)
			Cav1.3 (CACNA1D/Ca1d)
			Cav1.4 (CACNA1F/Ca1f)
P/Q-type	ω -Agatoxin	HVA	Cav2.1 (CACNA1A/Ca1a)
N-type	ω -conotoxin GVIA	HVA	Cav2.2 (CACNA1B/Ca1b)
R-type	SNX-482	Intermediate-voltage activated	Cav2.3 (CACNA1E/Ca1e)
T-type	None	LVA	Cav3.1 (CACNA1G/Ca1g)
			Cav3.2 (CACNA1H/Ca1h)
			Cav3.3 (CACNA1I/Ca1i)

Modified from Dolphin (Dolphin, 2009). HVA, high-voltage activated; LVA, low-voltage activated.

Table 2

Somatic and dendritic parameters of control and G93A-SOD1 motoneurons.

Parameter	Control (<i>n</i> = 38)	G93A-SOD1 (<i>n</i> = 39)
Soma area, μm^2	263.5 ± 9.2	299.3 ± 11.1
Soma perimeter, μm	90.2 ± 2.8	101.8 ± 3.9
Soma Feret diameter, μm	32.8 ± 1.1	35.1 ± 2.9
Dendritic length, μm	2285.4 ± 168.4	1989.2 ± 141.6 *
Number of branching points	67.4 ± 4.3	54.7 ± 4.5 *

Values are mean $\pm$ SEM. Statistical significance was determined using the Student's *t* test

* $p < 0.05$.

Table 3

Persistent Ca^{2+} current (PC_{Ca}) in control and G93A-SOD1 large Hb9-eGFP motoneurons.

	Control ($n = 52$)	G93A-SOD1 ($n = 44$)
V_{on} , mV	-43.7 ± 4.1	-41.7 ± 3.5
V_{off} , mV	-50.2 ± 4.8	-45.7 ± 4.5
V ($V_{\text{on}} - V_{\text{off}}$), mV (hysteresis)	7.2 ± 2.3	3.8 ± 1.8 *
I_{i} , pA	-407.2 ± 109.1	-632.3 ± 102.6 *
I_{s} , pA	-105.5 ± 21.8	-144.8 ± 27.2 *
I_{i}/C , pA/pF	-9.9 ± 2.4	-13.2 ± 2.7 *
I_{s}/C , pA/pF	-2.6 ± 0.6	-3.3 ± 0.7 *

Values are mean $\pm$ SEM. Statistical significance was determined using the Student's t test

* $p < 0.05$. See definitions in Fig. 1.

Table 4

Electrophysiological properties of control and G93A-SOD1 spinal motoneurons.

	Control (<i>n</i> = 10)	G93A-SOD1 (<i>n</i> = 9)
<i>Passive membrane properties</i>		
Resting membrane potential, mV	-68.5 ± 0.9	-67.8 ± 1.6
Input resistance, MΩ	50.4 ± 6.3	58.4 ± 5.6
<i>Action potential properties</i>		
Threshold, mV	-42.8 ± 0.5	-41.9 ± 0.8
Amplitude, mV	73.6 ± 4.3	69.1 ± 8.2
Duration, ms	1.7 ± 0.1	1.4 ± 0.3
AHP amplitude, mV	17.8 ± 1.5	16.2 ± 2.7

Values are mean ± SEM. Statistical significance was determined using the Student's *t* test

* *p* < 0.05. AHP, afterhyperpolarization.