Voltage-Dependent Sodium Channels in Spinal Cord Motor Neurons Display Rapid Recovery From Fast Inactivation in a Mouse Model of Amyotrophic Lateral Sclerosis

Cristina Zona, Massimo Pieri, and Irene Carunchio. Voltage-dependent sodium channels in spinal cord motor neurons display rapid recovery from fast inactivation in a mouse model of amyotrophic lateral sclerosis. J Neurophysiol 96: 3314–3322, 2006. First published August 9, 2006; doi:10.1152/jn.00566.2006. Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by a substantial loss of motor neurons in the spinal cord, brain stem, and motor cortex. Previous evidence showed that in a mouse model of a familial form of ALS expressing high levels of the human mutant protein Cu,Zn superoxide dismutase (Gly<sup>93</sup>→Ala, G93A), the firing properties of single motor neurons are altered to induce neuronal hyperexcitability. To determine whether the functionality of the macroscopic voltage-dependent Na<sup>+</sup> currents is modified in G93A motor neurons, in the present work their physiological properties were examined. The voltage-dependent sodium channels were studied in dissociated motor neurons in culture from nontransgenic mice (Control), from transgenic mice expressing high levels of the human wild-type protein [superoxide dismutase 1 (SOD1)], and from G93A mice, using the whole cell configuration of the patch-clamp recording technique. The voltage dependency of activation and of steady-state inactivation, the kinetics of fast inactivation and slow inactivation of the voltage-dependent Na<sup>+</sup> channels were not modified in the mutated mice. Conversely, the recovery from fast inactivation was significantly faster in G93A motor neurons than that in Control and SOD1. The recovery from fast inactivation was significantly faster in G93A motor neurons exposed for different times (3–48 h) and concentrations (5–500 μM) to edaravone, a free-radical scavenger. Clarification of the importance of these changes in membrane ion channel functionality may have diagnostic and therapeutic implications in the pathogenesis of ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder of unknown etiology. It occurs in familial (FALS) and sporadic (SALS) forms and is caused by a late-onset, progressive loss of motor neurons, leading to paralysis and death. FALS and SALS are distinguishable genetically, but not clinically. Nearly 20% of cases of FALS have been associated with >90 different mutations in the Cu,Zn superoxide dismutase enzyme (SOD1). These mutations are predominantly single amino acid replacements seemingly randomly scattered throughout the structure of this homodimeric 32,000-Da metalloprotein. Most of these mutant SOD1s essentially retain full activity, as measured in vitro, and there is overwhelming evidence in favor of a toxic gain of function as the cause of the disease (Borchelt et al. 1994; Bruijn et al. 1997; Reaume et al. 1996). The mechanisms by which mutations in SOD1 lead to motor neuron degeneration remain unidentified. Several mechanisms have been suggested by which a mutation of SOD1 may lead to motor neuron toxicity and degeneration, including the formation of intracellular aggregates (Johnston et al. 2000), production of reactive oxidative species (Estezvez et al. 1999), mitochondrial degeneration (Higgins et al. 2002), altered α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor permeability and subunit composition (Pieri et al. 2003b; Spalloni et al. 2004a), increase in intracellular Ca<sup>2+</sup> concentration by Ca<sup>2+</sup>-permeable AMPA receptor channels (Van Den Bosch et al. 2000; Weiss et al. 2000), and protein nitration (Casoni et al. 2005). Moreover, a mouse model of FALS described in previous papers showed neuronal hyperexcitability (Kuo et al. 2004; Pieri et al. 2003a). The relationship between the motor neuron’s firing frequency and injected current was altered, whereas the passive membrane properties were unaltered, indicating that FALS-like mutations of SOD1 induce alterations in the function of ion channels involved in membrane excitability.

The basis of spike generation in mammal spinal motor neurons is relatively constant during development. Na<sup>+</sup>-dependent action potentials are predominant from the onset of excitability (MacDermott and Westbrook 1986; McCobb et al. 1990; Ziskind-Conhaim 1988) and the activation of transient and sustained K<sup>+</sup> currents is observed during spike repolarization (Takahashi 1990). In the G93A mouse model of FALS, an increased persistent Na<sup>+</sup> current was observed (Kuo et al. 2005) that may contribute to the observed enhanced neuronal excitability (Kuo et al. 2004; Pieri et al. 2003a). In addition, a protective effect of TTX against kainate-induced toxicity was previously reported in G93A motor neurons but not in control and SOD1 neurons (Spalloni et al. 2004b). Taken together this evidence indicates an unequivocal involvement of the Na<sup>+</sup> channels in the model of ALS pathology.

Voltage-gated sodium channels are transmembrane proteins responsible for the rising phase of the action potential in the membranes of neurons and most electrically excitable cells. At resting membrane potential Na<sup>+</sup> channels are closed, but are rapidly activated by depolarizations (Catteral 1992). Activation of voltage-dependent Na<sup>+</sup> channels is produced by a voltage-driven change in the protein conformation that causes the ion pore to open, giving rise to Na<sup>+</sup> inward currents. These Na<sup>+</sup> currents decrease rapidly toward baseline levels as Na<sup>+</sup> channels undergo inactivation during prolonged depolarization.

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After inactivation, Na\(^+\) channels require repolarization to return to the resting state. Na\(^+\) channels in neuronal membranes are able to cycle through all these states on a millisecond timescale and are therefore able to mediate rapid events such as action potentials.

In this study we aimed to determine whether the physiological properties of the voltage-dependent Na\(^+\) channels are modified in spinal motor neurons obtained from a FALS model of ALS. Our results indicate that mutant SOD1 motor neurons have a modified Na\(^+\) channel functionality, indicating their involvement in the ALS pathology and that, by using the free-aldrac scavenger edaravone, this alteration does not arise from an altered function of SOD1 enzyme.

METHODS

Animal model

B6SJL-TgN (SOD1-G93A)1Gur mice expressing the G93A mutant SOD1 (G93A) and B6SJL-TgN (SOD1)2Gur mice expressing wild-type human SOD1 (SOD1), constructed by Gurney et al. (1994), were originally obtained from Jackson Laboratories (Bar Harbor, ME) and then housed in our animal facilities. Screening for the presence of the human transgene was performed on tail tips from adult mice and on the head from each embryo after removal of the spinal cord (Pieri et al. 2003b). Procedures involving animals and their care were conducted in strict accordance with the Policy on Ethics approved by the Society for Neuroscience and with the European Communities Council Directive for Experimental Procedures. Every effort was made to minimize the number of animals used and their suffering.

Primary dissociated culture

Mixed spinal cord cultures were prepared as previously described (Carriedo et al. 1996; Pieri et al. 2003b). Briefly, spinal cord cultures were prepared from 15-day-old embryos of a control female mated with a G93A male. Each neural tube was dissected and individually incubated for 10 min in 0.025\% trypsin and then dissociated by gentle triturate. The resulting mixed cultures were plated on poly-l-lysine–coated glass coverslips (four coverslips for each spinal cord) and maintained in DMEM supplemented with 5\% FBS and 5\% HS. By 2–3 h after plating, the medium was replaced with neurobasal supplemental with B-27, 0.5 mM glutamine, and 25 \( \mu \)M glutamic acid. To study the effect of free-radical scavengers, the cultured neurons were treated with different concentrations (5, 100, 500 \( \mu \)M) and hours (3, 6, 18, 24, 48 h) of exposure to 3-methyl-1-phenyl-2-pyrazolin-5-one (edaravone, Tocris Cookson, Bristol, UK). Edaravone was dissolved in dimethyl sulfoxide (DMSO) before being diluted to a final concentration of 0.1 \( \mu \)g/ml in the bath. Experiments were performed at room temperature (22–24°C). Recordings were obtained with an Axopatch 200B amplifier (Axon Instruments, Union City, CA). After the establishment of a gigaseal, the pipette resistance and capacitance were compensated electronically. After seal rupture, the whole cell capacitance was assessed on-line from the integral of the current transient after a 10-mV voltage step (membrane test function, pClamp 8; Axon Instruments). Cells with a capacitance of <25 pF were rejected, in accordance with the dimension criteria previously described, assuming 1 \( \mu \)F/cm\(^2\) as specific capacitance. Current signals were digitized at 50 kHz and filtered at 10 kHz. For long acquisitions the current signals were digitized at 10 kHz. Current density was calculated by dividing digitized current values by the whole cell membrane surface area estimated from whole cell capacitance measurements. P4 subtraction was used to eliminate capacitive transients and leak currents whenever possible.

Values of the voltage dependency of activation and inactivation were fitted with the Boltzmann equation

\[
\frac{I_{\text{max}}}{I} = 1 - [1 + \exp(V - V_{1/2})/\sigma]]
\]

where \( V \) is the potential of the given pulse, \( V_{1/2} \) is the potential for half-maximal activation and inactivation, and \( \sigma \) is the slope factor.

Inactivation time constants were determined using the Levenberg–Marquardt method to fit each trace with the following single-exponential equation

\[
I = A \times \exp(-\psi t) + C
\]

where \( I \) is the current, \( A \) is the relative proportion of the inactivated current with the time constant \( \tau \), and \( C \) is the steady-state noninactivating current. The time shift was selected manually as the point at which the macroscopic current began to inactivate exponentially.

The data related to the recovery from fast inactivation were fitted with the following exponential equation

\[
I_{\text{rel}} = A_{1} \times \exp(-t/\tau_{1})
\]

where \( I_{\text{rel}} \) is the amplitude of the current as a function of the time of recovery from inactivation, \( A_{1} \) is the relative percentage of current that recovered with the time constants \( \tau_{1}, t \) is the time, and \( I \) is the amplitude of the current related to the first depolarization.

Immunocytochemistry

SMI-32 staining (Sternberg Monoclonals; Carriedo et al. 1996) was performed. Cultures were fixed with 4\% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) for 30 min at room temperature, and then left for 20 min in blocking solution (2\% serum in PB with 0.3\% Triton X-100). Cultures were incubated in primary antibody (1:6,000 dilution) for 48 h at 4°C and visualized using a fluorescein cyanine-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA). Fluorescence images were acquired through a confocal laser scanning microscope (Zeiss, LSM 510, Oberkochen, Germany).
Data analysis

Fitting and statistical analysis were performed using Origin 6.1 (OriginLab, Northampton, MA) and MATLAB 6.0 for Windows (The MathWorks, Natick, MA). Parameter identifications were performed by the Nelder–Mead simplex method. The data were fitted with a number of iterations equal to 50. All fittings were accepted if the coefficient of determination ($R^2$) was $>0.98$ (De Coursey 2003). All results are expressed as means $\pm$ SD. The normality of the sample distribution and the homogeneity of the variances were tested by Kolmogorov–Smirnoff test and Leven’s test, respectively. The lack of differences was tested (two-way analysis) before to unit data from different cultures. ANOVA followed by Bonferroni’s test was used to determine the significance of the differences between Control, SOD1, and G93A. A $P < 0.05$ was considered significant.

RESULTS

Motor neurons were identified in primary culture by their morphological appearance (Fig. 1, A and B) and by their capacitance (see METHODS). To determine whether the overexpression of the mutated form G93A might cause alterations in the biophysical properties of voltage-activated sodium channels, we investigated these properties in Control, SOD1, and G93A motor neurons 8–13 days old in culture.

Voltage dependency of activation

The voltage dependency of activation was analyzed using a step protocol in which neurons were depolarized from a holding potential of $-80$ mV to a range of potentials from $-60$ to $+40$ mV in 5 mV increments. At potentials more positive than $-60$ mV, the voltage steps elicited rapidly activating inward currents that inactivated within 10 ms (Fig. 2A). These currents were blocked by 1 $\mu$M tetrodotoxin (TTX) (data not shown) and then defined as TTX-sensitive Na$^+$ currents. The currents in the three cell types are similar in voltage dependency, with a current first detectable at voltages near $-50$ mV and maximal near $-15$ mV. Peak currents for all neurons were normalized to the maximum peak current and plotted against voltage (Fig. 2B). In the three cell types, values of the means of the normalized peak current referring to the depolarizing voltage potentials were well fitted by a single Boltzmann function.
Voltages of the half-activation ($V_{1/2}$) and the slopes of the fitted curves were similar for the three neuronal populations (Fig. 2C).

To assess the differences between the amplitude of the currents of the three cell populations regardless of cell size, the voltage-dependent Na$^+$ current evoked at a test pulse of 10 mV for each motor neuron was normalized with respect to the whole cell surface area derived from measurements of the membrane capacitance (see METHODS). Also for the current density, no significant difference ($P > 0.05$) was observed in the three neuronal populations (Control: 45.17 ± 17.59 pA/pF, $n = 18$; SOD1: 36.52 ± 22.40 pA/pF, $n = 20$; G93A: 42.85 ± 11.13 pA/pF, $n = 23$).

Voltage dependency of steady-state inactivation and kinetics of fast inactivation

The voltage dependency of inactivation was determined using a two-step protocol in which a conditioning pulse was applied from a holding potential of −60 mV to a range of potentials from −90 to 0 mV in 10-mV increments for the duration of 40 ms, followed by a test pulse to +10 mV. Figure 3A shows the current traces related to Control, SOD1, and G93A motor neurons. The peak current amplitude during each test pulse was normalized to the current amplitude of the first conditioning pulse and plotted as a function of the conditioning pulse potential. The data shown in Fig. 3B indicate that the voltage dependency of steady-state inactivation was not significantly different for Control, SOD1, and G93A motor neurons in the $V_{1/2}$ or slope values obtained by fitting the values of the mean currents ($P > 0.05$).

To quantify the kinetics of fast inactivation, Na$^+$ current traces were recorded in a manner similar to that described for the voltage dependency of activation (Fig. 4A). The time constants were determined by fitting the decay phase of current traces with a single-exponential function, as described in METHODS, and were plotted against different test potentials. The tau values decreased with increasing test potentials, as shown in Fig. 4B. These values were not significantly different for Control, SOD1, and G93A neurons for each test potential ($P > 0.05$).

Recovery from fast inactivation

To analyze the recovery from fast inactivation, we used the two-pulse protocol. The protocol began with a conditioning depolarization from a holding potential of −80 to −10 mV for 10 ms, which inactivated the channels. This was followed by a second test pulse at the same depolarizing pulse value delivered to elicit Na$^+$ current fraction, whereas the time between the two pulses ($\Delta t$) varied between 0.5 and 45 ms, in 2-ms increments, to determine the rate of recovery. Figure 5A shows representative Na$^+$ current traces elicited using the described protocol in Control, SOD1, and G93A motor neurons. Fractional recovery for each neuron was calculated by dividing the current amplitude during the second test pulse by the current amplitude of the corresponding first conditioning pulse. The means with SD of these values for Control ($n = 12$), SOD1 ($n = 17$), and G93A ($n = 13$) motor neurons were plotted as a function of the time between the two pulses ($\Delta t$) and fitted with the exponential equation. The Control Na$^+$ channels recovered with a time course that was similar to that of SOD1 channels (3.81 and 3.74 ms, respectively), whereas the time course for G93A motor neurons decreased (2.33 ms) (Fig. 5B).

A significant statistical difference between G93A motor neurons and Control and SOD1 neurons was also observed when the time constants were calculated separately for each cell and then averaged, both in the experiments with the holding potential of −80 mV (Fig. 5C, left) and in the experiments with the holding potential of −60 mV (Fig. 5C, right).
To study whether free-radical scavengers were able to restore in G93A motor neurons the time of the recovery from inactivation to the normal values observed in Control and SOD1 neurons, we exposed the cultured cells to edaravone, a newly developed free-radical scavenger, in a time- and concentration-dependent manner (Watanabe et al. 1994; Zhang et al. 2005). The time of recovery from fast inactivation was not significantly modified compared with nontreated neurons when G93A cells were exposed to 5, 100, and 500 μM edaravone for 24 h (Fig. 6A). Similarly, when the G93A motor neurons were exposed for 3, 6, 18, 24, and 48 h to edaravone (100 μM), no significant difference was observable in the time of recovery from fast inactivation with respect to untreated neurons (Fig. 6B).

**Slow-inactivation entry, recovery, and voltage dependency**

In addition to the fast-gated properties examined above, sodium channels undergo slow-gated transitions that occur on a timescale of seconds to minutes. Therefore we examined the rate of entry into the slow-inactivated state, and the recovery from slow inactivation of G93A motor neurons, compared with SOD1 and Control motor neurons. The entry into the slow-inactivated state in the three neuronal populations was investigated using a conditioning pulse to −10 mV of 10 s (Fig. 7A) from a holding potential of −80 mV, followed by a 1-s recovery interval at −80 mV and by a depolarizing test pulse at −10 mV for 5 ms.

The interval at −80 mV allowed a virtually complete recovery of the channels from rapid inactivation. The fraction of Na⁺ current undergoing slow inactivation was derived from the ratio of Na⁺ current amplitude 1 s after the conditioning pulses to Na⁺ current amplitude of the first depolarizing pulse. The fraction of available Na⁺ current was not significantly different \((P > 0.05)\) for Control \((0.63 \pm 0.12, n = 6)\), SOD1 \((0.64 \pm 0.12, n = 5)\), and G93A motor neurons \((0.61 \pm 0.06, n = 5; \text{Fig. } 7A)\).

To assess the process of recovery from the slow inactivation, the same stimulation protocol was applied, followed by a series of brief (5 ms) test pulses to −10 mV at a frequency of 0.33 Hz intended to monitor the process of recovery from slow inactivation (Fig. 7B). When the current amplitude reached steady-state values, the pulse protocol was terminated. The current amplitudes were normalized to the amplitude of the first depolarizing pulse. At the prepulse duration tested (10 s), there was no significant difference in the process of recovery from slow inactivation of Control \((n = 11)\), SOD1 \((n = 10)\), and G93A \((n = 11)\) motor neurons (Fig. 7B).

We next tested whether Na⁺ current slow inactivation in Control, SOD1, and G93A motor neurons was different in terms of voltage dependency. We applied 5 s conditioning pulses from −100 mV to different conditioning potentials ranging from −100 to +20 mV and monitored the fraction of Na⁺ currents recovered from slow inactivation after 1 s using a 5 ms test pulse to −10 mV (Fig. 7C). Our results show that slow inactivation was clearly dependent on the voltage of the conditioning pulse, resulting in a profound reduction of the available current with more depolarizing conditioning potentials. For each depolarizing conditioning potential there was no significant difference between Control \((n = 6)\), SOD1 \((n = 5)\), and G93A \((n = 5)\) motor neurons (Fig. 7C).

**DISCUSSION**

These results provide the first evidence that G93A mice, in an early and probably presymptomatic phase, have already developed an alteration of the fast transient voltage-gated Na⁺ channel functions. Our results show that in these mice the recovery from fast inactivation is accelerated compared with
Control and SOD1, whereas the kinetics of activation and that of slow inactivation do not appear to be modified. This reinforces the hypothesis that the changes observed in the functional properties of the Na\(^+\)/H\(^+\) channel in the motor neurons from transgenic G93A mice specifically arise from the expression of the SOD1 mutation associated with the disease.

The hypotheses of oxidative stress in the ALS pathogenesis have arisen predominantly from studying the effects of SOD1 mutations. Mutant SOD1 produces motor neuron injury by a toxic gain of function and, although the exact mechanism of action is unclear, several hypotheses exist, including aberrant free-radical handling (Yim et al. 1996). To test whether the modified functionality of the Na\(^+\)/H\(^+\) channels in G93A motor neurons is the result of an altered function of SOD1, an enzyme that is part of cellular antioxidant defense systems, we tested edaravone, a free-radical scavenger (Watanabe et al. 1994; Zhang et al. 2005). The exposure of G93A motor neurons to this free-radical scavenger, in a time- and concentration-dependent manner, did not induce any effect on the time of recovery from the fast inactivation in G93A motor neurons, indicating that the altered Na\(^+\)/H\(^+\) channel functionality was not dependent on the lack of function of the mutant SOD1 free-radical scavenger.

**FIG. 5.** Recovery from fast inactivation. A: representative Na\(^+\) current traces obtained using the protocol shown in inset to study the recovery from fast inactivation in Control, SOD1, and G93A motor neurons 12, 12, and 13 days old in culture, respectively. B: plot of recovered current against lengths of delay of \(\leq 45\) ms between depolarizing steps. All points represent data from Control \((n = 12)\), SOD1 \((n = 17)\), and G93A \((n = 13)\) motor neurons. Recoveries were fitted with one exponential with the G93A tau value that differed from Control and SOD1. C: histogram of the time constant means with SD for Control \((12.58 \pm 2.5\) ms, \(n = 9)\), SOD1 \((12.9 \pm 3.04\) ms, \(n = 6)\), and G93A \((8.81 \pm 0.28\) ms, \(n = 12)\) motor neurons with a holding potential of \(-80\) mV \((left)\) and for Control \((12.58 \pm 2.5\) ms, \(n = 9)\), SOD1 \((12.9 \pm 3.04\) ms, \(n = 6)\), and G93A \((8.81 \pm 0.28\) ms, \(n = 12)\) motor neurons with a holding potential of \(-60\) mV \((right)\). Means are significantly decreased for G93A neurons compared with Control and SOD1 in both experimental conditions.

**FIG. 6.** Effect of edaravone on the recovery from fast inactivation in G93A motor neurons. A: recoveries from fast inactivation were fitted with one exponential in G93A neurons of Control and in G93A neurons treated with 5, 100, and 500 \(\mu\)M edaravone for 24 h in the culture medium. Tau means in 5 \(\mu\)M \((2.39 \pm 0.54, n = 5)\), 100 \(\mu\)M \((2.27 \pm 0.51, n = 6)\), and 500 \(\mu\)M \((2.37 \pm 0.65, n = 5)\) treated neurons were not significantly different in comparison to untreated G93A motor neurons \((2.39 \pm 0.52, n = 8)\). B: histogram of the time constant means with SD for untreated G93A motor neurons \((2.39 \pm 0.52, n = 8)\) and G93A neurons exposed to 100 \(\mu\)M edaravone for 3 h \((2.41 \pm 0.55, n = 6)\), 6 h \((2.45 \pm 0.73, n = 5)\), 18 h \((2.43 \pm 0.39, n = 5)\), 24 h \((2.27 \pm 0.51, n = 6)\), and 48 h \((0.42 \pm 0.39, n = 7)\). Means are not significantly different. All recordings were performed on motor neurons 8–9 days old in culture.
Ion channel involvement in ALS pathology has now become clear. Previous papers reported a functional alteration of the voltage-dependent Na$^+$ channel in mutant G93A transfected neuroblastoma cells SH-SY5Y (Zona et al. 1998), a modified permeability and composition of the AMPA receptors in the G93A familial model of ALS (Pieri et al. 2003b; Spalloni et al. 2004a) and, in the same model, a modification in striatal permeability and composition of the AMPA receptors in the neuroblastoma cells SH-SY5Y (Zona et al. 1998), a modified fraction of Na$^+$ channels. Fraction of available Na$^+$ current was not significantly different ($P > 0.05$) for Control ($0.63 \pm 0.12, n = 6$), SOD1 ($0.64 \pm 0.12, n = 5$), and G93A motor neurons ($0.61 \pm 0.06, n = 5$). $B$: recovery from slow inactivation. Number of available Na$^+$ channels after slow recovery from inactivation is similar in Control, SOD1, and G93A motor neurons. Current amplitude during recovery was normalized to the current related to the first depolarization, yielding the fraction of available Na$^+$ channels. Normalized values are plotted vs. the recovery time for the indicated prepulse duration, no significant difference being found in the 3 populations. $C$: voltage dependency of Na$^+$ current slow inactivation. Fraction of Na$^+$ channels available 1 s after the depolarizing conditioning pulse is plotted vs. the prepulse potential in Control, SOD1, and G93A motor neurons. For all the values of the depolarizing conditioning pulse, there is no significant difference ($P > 0.05$).

FIG. 7. Na$^+$ channels entry into and recovery from the slow inactivation. $A$: fraction of Na$^+$ channels available after a depolarization of 10 s followed by a recovery of 1 s in Control, SOD1, and G93A motor neurons. Second test pulse had a duration of 5 ms. Current amplitude after 1 s of recovery was normalized to the current related to the first depolarizing pulse, yielding the fraction of available Na$^+$ channels. Fraction of available Na$^+$ current was not significantly different ($P > 0.05$) for Control ($0.63 \pm 0.12, n = 6$), SOD1 ($0.64 \pm 0.12, n = 5$), and G93A motor neurons ($0.61 \pm 0.06, n = 5$). $B$: recovery from slow inactivation. Number of available Na$^+$ channels after slow recovery from inactivation is similar in Control, SOD1, and G93A motor neurons. Current amplitude during recovery was normalized to the current related to the first depolarization, yielding the fraction of available Na$^+$ channels. Normalized values are plotted vs. the recovery time for the indicated prepulse duration, no significant difference being found in the 3 populations. $C$: voltage dependency of Na$^+$ current slow inactivation. Fraction of Na$^+$ channels available 1 s after the depolarizing conditioning pulse is plotted vs. the prepulse potential in Control, SOD1, and G93A motor neurons. For all the values of the depolarizing conditioning pulse, there is no significant difference ($P > 0.05$).
These channels display two modes of inactivation: fast and slow. Fast inactivation describes the rapid decay of Na\(^+\) currents observed in response to short depolarizations. Slow inactivation occurs when neuronal cells are depolarized for seconds or minutes. Slow and fast inactivations are not only kinetically distinct, they also involve different structural elements (Ellerkmann et al. 2001). Fast inactivation of Na\(^+\) channels ensures that the action potentials are brief, but this also creates a refractory period during which neurons cannot fire action potentials. The duration of this refractory period depends on the time necessary for the inactivated Na\(^+\) channels to move back to the resting state after the repolarization of the membrane (Kuo and Bear 1994).

The orchestrated activation and inactivation gating of sodium channels is vital to normal neuronal signaling. Even small syncopations in this normal gating rhythm may alter cellular excitability, leading to many different disorders. In fact, alterations in the Na\(^+\) channel kinetics gating were previously observed in many neuropathologies (Cummins et al. 1993; Green et al. 1998; Meisler and Kearney 2005; Mitrovic et al. 1995; Rook et al. 1999). Febrile convulsions and epilepsy are associated with a mutation in the gene encoding the \(\beta\)-subunit of the human neuronal Na\(^+\) channel and mutations in the gene encoding the \(\alpha\)-subunit have been shown to result in hyperexcitability by the mechanism of more rapid recovery from inactivation (Chen et al. 1998; Hayward et al. 1996).

Neurons with Na\(^+\) channels that recover rapidly from inactivation are able to fire at higher frequencies (Torkkeli and French 2002), resulting in hyperexcitability, as in the G93A model of FALS (Kuo et al. 2004; Pieri et al. 2003a). High-frequency firing may lead to a large and simultaneous increase in the free intracellular concentrations of sodium and calcium, which would then render the Na\(^+\)/Ca\(^{2+}\) exchanger ineffective in the fast extrusion of calcium (Reuter and Porzig 1995).

The functional alteration in the recovery from inactivation of the Na\(^+\) channels observed in G93A motor neurons may reflect a number of factors, such as the existence of multiple \(\alpha\)- and \(\beta\)-subunits having different properties and being differently expressed, the different channel modulation induced by endogenous compounds, transcriptional alterations, and indirect effectors whose functions could be modified in the presence of mutant SOD1. Each of these factors can contribute to the modified functionality of the Na\(^+\) channel expressed in the G93A motor neurons and further experimental evaluation will be necessary.

In conclusion, we have shown that G93A motor neurons present a significantly faster recovery from fast inactivation when compared with SOD1 and Control motor neurons, whereas the density of Na\(^+\) currents, the curve of activation and inactivation, and the entry and recovery from slow inactivation appear unchanged. The altered functionality of the Na\(^+\) channels was still present in the neurons treated with a free-radical scavenger, indicating that the changed parameter was not dependent on the lack of function of the mutated SOD1. This alteration, demonstrated at the single-cell level, may affect the normal orchestrated activation and inactivation gating of the voltage-dependent channels involved in the neuronal excitability and may be significant in the onset and progression of the pathology. In this context, the finding of an intrinsic functional alteration of Na\(^+\) channels indicates the necessity for further research in this area. State-dependent modulation of Na\(^+\) channels may provide a mechanism for selective drug action under pathological conditions. In the future, for clinical purposes, it will be important to analyze the action mechanisms of putative compounds, especially those interacting with the fast inactivation of Na\(^+\) channels.

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