Hyperexcitability of Cultured Spinal Motoneurons From Presymptomatic ALS Mice

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Hyperexcitability of cultured spinal motoneurons from presymptomatic ALS mice. J Neurophysiol 91: 571–575, 2004. First published October 1, 2003; 10.1152/jn.00665.2003. ALS (amyotrophic lateral sclerosis) is an adult-onset and deadly neurodegenerative disease characterized by a progressive and selective loss of motoneurons. Transgenic mice overexpressing a mutated human gene (G93A) coding for the enzyme SOD1 (Cu/Zn superoxide dismutase) develop a motoneuron disease resembling ALS in humans. In this general accepted ALS model, we tested the electrophysiological properties of individual embryonic and neonatal spinal motoneurons in culture by measuring a wide range of electrical properties influencing motoneuron excitability during current clamp. There were no differences in the motoneuron resting potential, input conductance, action potential shape, or afterhyperpolarization between G93A and control motoneurons. The relationship between the motoneuron’s firing frequency and injected current (f-I relation) was altered. The slope of the f-I relation and the maximal firing rate of the G93A motoneurons were much greater than in the control motoneurons. Differences in spontaneous synaptic input were excluded as a cause of increased excitability. This finding identifies a markedly elevated intrinsic electrical excitability in cultured embryonic and neonatal spinal G93A motoneurons. We conclude that the observed intrinsic motoneuron hyperexcitability is induced by the SOD1 toxic gain-of-function through an aberration in the process of action potential generation. This hyperexcitability may play a crucial role in the pathogenesis of ALS as the motoneurons were cultured from presymptomatic mice.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an adult-onset and deadly neurodegenerative disease characterized by a progressive and selective loss of motoneurons (Cleveland and Rothstein 2001). Mutations in the enzyme SOD1, which is involved in detoxification of free radicals, account for ~20% of the familial form of ALS (Rosen et al. 1993). Transgenic mice overexpressing the mutated human SOD1 gene develop phenotypic and pathological symptoms resembling ALS in humans (Gurney et al. 1994; Siddique and Deng 1996), including the progressive development of muscle weakness leading to paralysis (Kong and Xu 1998). Studies using these transgenic mice and SOD1 knockout mice suggest that the motoneuron degeneration does not result from a loss of SOD1 enzyme activity but from a toxic effect induced by the presence of mutant SOD1 itself (“toxic gain-of-function”) (Gurney et al. 1994; Reaume et al. 1996; Wong et al. 2002). Several unique pathways possibly involved in selective motoneuron degeneration have been identified in SOD1 mice, but the initiating mechanism(s) underlying this process have not yet been defined (Hand and Rouleau 2002).

Surprisingly, the intrinsic electrical properties of motoneurons themselves have largely been ignored. We hypothesized that an altered excitability of spinal motoneurons in the presymptomatic period may play a fundamental role in the observed deficits of the ALS model mouse. We, therefore compared the intrinsic electrical properties of motoneurons cultured from embryonic and neonatal transgenic SOD1 (G93A) mice to control mice, using complementary culturing and stimulation techniques.

METHODS

From neonatal organotypic spinal cord slice cultures (NC; UMC Utrecht), intracellular recordings were made using sharp microelectrodes and the relationship between the motoneuron’s firing frequency and injected current (f-I relation) was measured on the transient unadapted motoneuron responses to short current pulses. From embryonic primary spinal cord cell cultures (EC; Northwestern), motoneuron recordings were made with patch-clamp electrodes. A slow triangular current stimulation was applied to these neurons so that their f-I relation was determined on a steady-state train of action potentials with a much adapted firing rate. These two complementary stimulus protocols were chosen such that the generated responses covered the whole functional range of motoneuron activation. In both preparations, mice expressed a high copy number of the glycine to alanine mutation at base pair 93 of the SOD1 gene (The Jackson Laboratory, Bar Harbor, ME, USA). Transgenic progeny were identified using standard polymerase chain reaction (PCR) techniques (Rosen et al. 1993). The mice began to demonstrate clinical symptoms at 90 (1Gur; NC) and 200 days of age (1Gur; EC).

In the NC studies, mice were backcrossed to the Balb/c background (Harlan, Zeist, The Netherlands), and nontransgenic G93A littermates were the control animals. All procedures were approved by the Utrecht University Committee for Experiments on Animals. The slice cultures, which have been shown previously to maintain a healthy motoneuron population over several months, were pre-

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pared from 7-day-old mice (see Bär 2000; Kaal et al. 2000 for details). After ~3 wk in culture, the slices were placed in a recording chamber perfused with artificial cerebrospinal fluid (ACSF; containing in mM: 127 NaCl, 1.9 KCl, 1.2 KH2PO4, 1.3 MgSO4, 20 NaHCO3, 2.4 CaCl2, and 10 glucose; gassed with 95% O2-5% CO2; pH 7.4). Intracellular recordings were made, at room temperature using glass electrodes filled with 3 M KCl (60–90 MΩ), from 39 motoneurons (23 control; 16 G93A; 1 motoneuron per cultured slice). Motoneurons were identified by their location, appearance, and diameter (>25 μm). Membrane input resistance was measured using small rectangular hyperpolarizing current pulses. A series of rectangular depolarizing current pulses with increasing amplitude (150-ms duration, 1 Hz; see Fig. 1A) was used to determine motoneuron excitability.

In the EC studies, control animals were transgenic mice expressing the human wild-type SOD1 gene at similar levels. Breeding and PCR identification of control mice were identical to the G93A mice. The primary cell cultures of spinal neurons were generated from embryonic mice (day 12–14) using modified techniques described in Anelli et al. (2000). All procedures were approved by the Northwestern University animal care and use committee. Motoneurons were selected based on soma diameter (Carriedo et al. 1995) and expressed as instantaneous frequency. Differences in measurement techniques between the slice and cell cultures are noted in the following text.

In the EC motoneurons, AP properties were measured using the stimulus intensity that elicited a single AP in the f-I protocol. The membrane resistance, AP properties, and firing threshold were quantified by averaging values obtained on at least four responses. The interval between the first and second AP was measured (average of 4) and expressed as instantaneous AP frequency. The input-output relation was calculated by plotting the instantaneous AP frequency against the amplitude of the stimulating current (see Fig. 1B). The f-I relations were fit (Microlab Origin) with an exponential growth function: spike rate = fmax{1 − exp[−(I − Ith)]} to obtain values for maximum firing rate (fmax), current amplitude at firing threshold (Ith, or I0) and slope (k InternalSynapticTransmission) at threshold. The amplitude of the AHP after the AP train (posttrain AHP) evoked by the 150-ms current pulse, was expressed in mV/spike. This value represents the mean contribution of an individual AP to the AHP as obtained from the linear relation between the number of APs in a train and the AHP size. The AHP decay time constants were measured as the time required for the voltage to reach 63% of the asymptotic value.

In the NC motoneurons, input resistance was calculated by fitting a linear regression to the current-voltage relation, subthreshold to the onset of a persistent inward current (Powers and Binder 2001). All AP properties were measured using the first AP in the spike train. The steady-state f-I gain was determined by fitting a linear regression line to the instantaneous firing frequency versus the corresponding change in current. AP thresholds were calculated as the voltage at 0.5 ms before the peak of the second derivative.

FIG. 1. Traces of evoked action potentials and the relations between the motoneuron’s firing frequency and injected current (f-I relations) in neonatal organotypic spinal cord slice cultures (NC; A and B) and embryonic primary spinal cord cell cultures (EC; C and D) motoneurons. A: voltage response (−70 mV) to depolarizing current pulses of increasing amplitude. The interval between the 1st and 2nd spike was expressed as instantaneous frequency. B: the f-I relation was fit with an exponential growth function (see Methods), fmax: maximum firing rate, Ith: current amplitude at firing threshold. C: voltage response to depolarizing current ramp protocol. Instantaneous spike frequency was determined. D: the f-I relation was fit with a linear regression, from which the slope (gain) was measured.
rates between NC and EC cells are accounted for by recording techniques and applied current stimuli (Fig. 1), respectively. Regression analysis showed that hyperexcitability is not due to input resistance.

**METHODS**

Comparison of excitability of G93A (neonatal) and control motoneurons. Values are means ± SD. Neonatal (NC) neurons were held at −70 mV by DC current clamp to prevent generation of action potentials by the spontaneous excitatory synaptic activity. Differences between mean values were determined by t-test. Significance is indicated by * (P < 0.05), ** (P < 0.005), and *** (P < 0.0005). The difference between the embryonic (EC) G93A and control spike amplitude is unlikely to affect the excitability, as there was no correlation (r² = 0.0001) between excitability (f-I slope) and spike amplitude. NM, not measured; AHP, afterhyperpolarization.

**RESULTS**

In both neonatal (NC) and embryonic (EC) cultured motoneurons, no differences were observed between SOD1 (G93A) motoneurons and controls with respect to motoneuron appearance, soma diameter, resting membrane potential, and input resistance (Table 1). AP properties were similar for G93A and control neurons (Table 1). The f-I relations for the NC cells were obtained from the voltage responses to current pulses of variable amplitude (duration: 150 ms; Fig. 1A). The f-I relations (Fig. 1B) were fit with an exponential function (see **METHODS**) to obtain the maximum firing rate (f\text{max}), current amplitude at firing threshold (I\text{th}), and slope (at I\text{th}). In the EC cells, f-I relations were generated using slow ramps of injected current (Fig. 1C) with the gain defined as the slope (Fig. 1D). I\text{th} was the current amplitude at the first spike. Results for motoneurons in both types of preparations were similar. No differences were found for I\text{th} between the controls and G93A neurons in either data set (Table 1). The slope of the f-I curves, though, was markedly steeper (P < 0.005) for the G93A neurons (Fig. 2, A and B; Table 1). The difference in slope between the f-I curves of NC and EC motoneurons results from the different degree of adaptation in the firing rates induced by the two stimulus protocols (see **METHODS**). Despite the differences in G93A expression level, preparations, procedures, and control mice, the increase in f-I gain was similar (1.5- and 1.7-fold for EC and NC cells, respectively). Moreover, in the NC motoneurons, f\text{max} was also larger (P < 0.005) for the G93A than the control neurons (Fig. 2C; Table 1).

One explanation for this elevated excitability would be a smaller or faster decaying postspike AHP in the G93A neurons because the AHP is involved in regulating the AP afterhyperpolarization.

**FIG. 2.** Comparison of excitability of G93A (○) and control (□) motoneurons as a function of input resistance. A: gain of NC (neonatal) motoneurons. B: gain of EC (embryonic) motoneurons. C: f\text{max} of NC motoneurons. Differences in resistance and firing rates between NC and EC cells are accounted for by recording techniques and applied current stimuli (Fig. 1), respectively. Differences between mean values were determined by t-test. Significance is indicated by ** (P < 0.005) and *** (P < 0.0005). Regression analysis showed that hyperexcitability is not due to input resistance.
The presence of blockers of ionotropic receptors in the entire EC data set precludes the excitability difference resulting from spontaneous transmitter release from interneurons (Rekling et al. 2000). Moreover, the f-I relation of a subset of NC control motoneurons (n = 5) was unaffected by a similar (see methods) blockade of synaptic input, applied subsequent to control measurements. The addition of the receptor blockers did eliminate oscillations and synaptic noise in the membrane potential but did not affect the passive electrical or AP properties of the motoneurons (not shown), including the gain (control: 0.22 ± 0.09 spikes · s⁻¹ · PA⁻¹; blockers: 0.19 ± 0.07; P > 0.5) and \( f_{\text{max}} \) (101 ± 33 vs. 103 ± 42 spikes/s; \( P > 0.9 \)) of their f-I relation. From these findings, we conclude that the higher gain and \( f_{\text{max}} \) demonstrate an intrinsic elevated electrical excitability of the EC and NC motoneurons in ALS mice.

DISCUSSION

Our measurements provide evidence, obtained at the cellular level, that SOD1 mice in an early and probably presymptomatic phase have already developed a functional motoneuron aberration. This aberration presumably results from the SOD1 toxic gain-of-function. Our results suggest that the hyperexcitability of the motoneuron membrane reflects an intrinsic aberration of the motoneuron because synaptic transmission was blocked and no changes in the AHP were observed. This conclusion is supported by reported deviations in Na⁺ currents (Zona et al. 1998) in G93A-transfected human neuroblastoma cells. The ionic mechanism underlying enhanced firing has yet to be elucidated but may involve the persistent Na⁺ current, which appears to be important in setting the motoneuron Na⁺ current (Lee and Heckman 2001). In fact, an increased persistent Na⁺ current in cultured SOD1 (G93A) motoneurons has been reported in a preliminary study (Kuo et al. 2002). Taken together, these results strongly suggest that the hyperexcitability is caused by an aberration in the process of AP generation. Further, an increase in persistent Na⁺ conductance has also been suggested in a clinical neurophysiological study measuring the strength-duration properties of sensory and motor axons (Mogyoros et al. 1998). In this study, motor axons from ALS patients exhibited characteristics of increased persistent Na⁺ conductance, whereas sensory axons did not. Moreover, these observations are consistent with a neuroprotective property, selective inhibition of persistent Na⁺ at therapeutic concentrations, of the ALS treatment drug riluzole (Urbani and Belluzzi 2000).

The motoneurons studied were cultured (for 2–4 wk) from embryonic and neonate presymptomatic mice. Although cellular development in culture is not directly comparable to in vivo, the cultured cells were studied at a time point much earlier than the age at which the SOD1 mice begin demonstrating clinical deficits (≥3–4 mo) (Gurney et al. 1994). Moreover, pathological signs of motoneuron degeneration, such as aggregates (Bruijn et al. 1998), mitochondrial dysfunction (Kong and Xu 1998), and caspase activation (Li et al. 2000), do not appear prior to 2 mo postnatal. At the time point of 2 mo postnatal, a significant peripheral axonal degeneration has been reported to occur in the SOD1 mouse (Frey et al. 2000). With respect to the motoneuron soma, however, (from which our recordings were made) cytoskeletal abnormalities in G93A high expressor mice (onset: 90 days) could be observed (Tu et al. 1996) at 82 days but not at 45 days postnatal. The increased excitability we report here was not accompanied by changes in other basic electrical properties of the SOD1 motoneurons. Also, during the 2–3 wk of culturing no obvious differences were observed in culture survival and in motoneuron appearance. This together is thought to imply that the EC and NC motoneurons were essentially healthy at the time point that they were studied and probably in a presymptomatic condition. It should be mentioned, however, that an extrapolation of the potential significance of these results to in vivo conditions is somewhat limited by the cultured nature of the motoneurons studied.

Interestingly, an elevation of cortex motoneuron excitability in a very early stage of ALS has been suggested by several studies applying a variety of noninvasive in vivo techniques to ALS patients (Eisen and Weber 2001). In a recent study of particular importance (Zanette et al. 2002), an increased gain of the dose-response curves to transcranial magnetic stimulation in ALS patients was reported. This increased gain is thought to result from an elevated excitability of the corticomotoneurons, therefore contributing to motor cortex hyperexcitability (Zanette et al. 2002).

The enhanced number of APs induced in the soma of SOD1 motoneurons during activation, resulting from hyperexcitability, can contribute to the various pathological mechanisms proposed for motoneuron degeneration (Cleveland and Rothstein 2001; Hand and Rouleau 2002; Wong et al. 2002). For instance, enhanced firing rates can contribute to the development of oxidative stress (Lancelot et al. 1998) and will increase the load on mitochondrial metabolism (Heath and Shaw 2002), which probably plays a key role in selective motoneuron death (Kaal et al. 2000). In addition, because the activity of the membrane Na/K pump is severely reduced in the spinal cord of SOD1 mice (Ellis et al. 2003), increased firing rates will contribute to disturbances of the cell’s ion metabolism. Because ALS vulnerable spinal motoneurons have been shown to poorly buffer Ca²⁺ (Palecek et al. 1999) in comparison to ALS-resistant motoneurons (Vanselow and Keller 2000), the increased Ca²⁺ influx due to hyperexcitability may play a critical role in selective motoneuron degeneration.

The finding of a motoneuron aberration in an early and probably presymptomatic phase of ALS suggests that hyperexcitability may be present in presymptomatic human patients. Overall, the enhanced motoneuron excitability provides an alternative viewpoint for the mechanism of selective motoneuron degeneration and reveals a possible link to previously published observations during the development of ALS (Zanette et al. 2002).

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