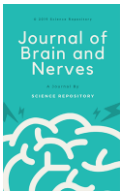

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Research Article

Electrophysiological and Morphological Studies of *SOD1* Transgenic Mice: An Animal Model of ALS

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a disease where upper and lower motor neurons die, and it is often associated with mutations of superoxide dismutase 1 (*SOD1*). We have used mouse models to compare physiologic and morphologic characteristics of cervical motor neurons in wild-type and mutant animals. Slices of the cervical spinal cord were prepared from old wild-type and mutant *G93A* and *G85R* mice, and intracellular recordings of membrane potential, resistance and responses to application of excitatory neurotransmitters were studied. Some motor neurons were injected with Lucifer Yellow for morphological analysis. There were no significant differences between membrane potential in the *SOD1* mutants and aged wild-type mice, but membrane resistance was somewhat higher in the mutant motor neurons. Dendrites of the mutant motor neurons were not responsive to ionophoretic application of excitatory amino acids, although the cell body responded strongly. In Lucifer-filled cells, the dendrites were found to disappear. Mutant motor neurons were sometimes spontaneously active. Responses of mutant motor neurons to perfused glutamate with varying calcium concentrations in the Ringer's solution were different from those of the wild-type cells.

Introduction

In 1869, French neurologist, Jean-Martin Charcot discovered a disease in humans characterized by abnormalities and loss of skeletal muscle mass and named it amyotrophic-related lateral sclerosis (ALS) [1]. Most ALS is sporadic, but 5 to 10% is familial. Some 20 different mutations have been identified in familial ALS [2]. Mutations of superoxide dismutase 1 (*SOD1*) have been seen in about 20% of familial cases and 3% of sporadic cases [3, 4]. Mouse models of these mutations have been developed; the best studied being *SOD1-G93A* [5]. In the *SOD1* mutant mouse, the muscle force begins to decline around about 150 days after birth [6].

Biochemical observations of the *SOD1* mutant mice show mitochondrial dysfunction, SOD1 aggregation, motor neuron cell death and paralysis [7]. There is a striking increase in oxidative stress, as in common among other neurodegenerative diseases [8]. A major role of mitochondria is calcium regulation, and this is clearly disrupted in ALS. Reactive oxygen species (ROS) are generated by the intracellular influx of a large quantity of calcium going through both voltage and agonist activated calcium channels, and these processes play a major role in motor neuron cell death. Homeostasis of the concentration of intracellular calcium is lost [9]. There is also a fragmentation of the Golgi apparatus, which further disrupts cell function [10].

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Glutamate is the major excitatory neurotransmitter in the central nervous system. There are several different glutamate receptors (AMPA, kainic acid and NMDA), but the AMPA receptor is the one responsible for usual synaptic transmission. This receptor usually consists of four subunits, GluA1, GluA2 GluA3 and GluA4), and the open ion channel is not permeable to calcium [11]. However, without RNA editing of GluA2, the AMPA receptor is permeable to calcium. Kwak and Kawahara (2005) have shown both in mice and humans that ALS is associated with abnormal editing of GluA2 and have provided support for the hypothesis that the motor neuronal cell death is due to elevation in calcium concentration [12, 13]. We have performed electrophysiologic and morphologic studies comparing wild-type cervical motor neurons to those of *SOD1* mutant mice in order to investigate changes occurring in structure, membrane biophysical properties and responses to excitatory amino acid neurotransmitters during the period in which motor neurons are showing injury.

Methods and Materials

We studied motor neurons in the cervical spinal cord of 55 *G93A* mutant mice, 20 *G85R* mutant mice and 22 wild-type mice. We used methods described in previous studies on rat spinal cord and those described for mouse in the accompanying paper [14, 15]. The vertebra surrounding the spinal column of mice is not as hard as in the rat and can be removed without damaging the spinal cord. It is also easier to remove remnants of the surrounding dura mater in comparison with an albino rat. The size of a mouse is smaller than a rat, and also the skeleton is thinner. We perfused cold Ringer's solution over the cord during dissection, which significantly reduced the rate of metabolism. Only motor neurons were studied that had a membrane potential of about -60 mV and for which stable recordings were maintained for a period of at least 20 min.

As already reported, we removed an approximate 7 mm length of the cervical spinal cord around the biggest part (C-5), and we prepared slices of about 450-microns [14, 15]. The slices were preincubated in a modified Krebs-Ringer solution for about one hour. The composition of the modified Krebs-Ringer solution was 212.5 mM sucrose, 3.5 mM KCl, 2.4 mM CaCl, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 10 mM glucose saturated with 95% O_2/5% CO_2 [16]. During the recording, the composition of the Krebs-Ringer solution was 125 mM NaCl, 3.5 mM KCl, 2.4 mM $CaCl_2$, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 1.2 mM KH_2PO_4 saturated with 95% O_2/5% CO_2. Recordings were carried out in a submerged recording chamber perfused by normal Krebs-Ringer solution circulating at about 5 ml/min at 32˚C. Electrical membrane properties of motor neurons were measured in the slice preparations by using conventional glass microelectrodes connected to a DC amplifier (Neuro Data) with a bridge circuit [14]. The glass micropipettes were made from borosilicate capillary tubing was filled with 3 M potassium acetate and having a resistance of about 150 MΩ.

Chemicals were added to the perfused Ringer's solution or were placed in a glass micropipette and applied into the dendrite trees of the motor neuron ionophoretically. The chemicals used in these studies were excitatory amino acid agonists. Glutamate, AMPA and kainic acid (KA) were prepared as a 10 mM solution in 0.15 M NaCl in pH 7.5. For morphological analysis, microelectrodes were filled with Lucifer yellow CH (Sigma, 10% in distilled water) and were injected into motor neurons in the slices by passing current (2 nA negative current pulses of 250 ms at 2 Hz for 1,5-2 min). All studies of both *SOD1* mutants and wild-type controls were done on mice at about post-natal day (PD) 125 to PD250. At these ages, both of the *SOD1* mutant mice were showing a significant decrease in muscle force [6]. To the degree possible we compared mutant and wild-type responses in animals as close as possible to the same ages. We observed no significant differences in results obtained from the *G93A* as compared to the *G85R* mutant motor neurons, and therefore for some results, we pooled the mutant data for comparison with the wild-type.

Results

Figure 1 shows membrane potential and membrane resistance from motor neurons recorded from each mutant animal and their wild-types. Input resistance was significantly higher in the motor neurons from both mutant mice, although there was no significant difference between mutant and wild-type membrane potential. Figure 2 shows the structure of cervical motor neurons from wild-type mice at ages between PD150 to PD215 and of *G93A* mutant mice at ages PD143 to PD306. Wild-type motor neurons show extensive dendritic trees, but for the mutant animals, there is a large loss of dendrites with age. At PD306 there are effectively no dendrites left. Figure 3 shows responses to ionophoretic applications of excitatory amino acid neurotransmitters onto motor neurons from wild-type and *G93A* animals. The ionophoretic electrode, which was filled with neurotransmitter, was placed about 300 μm away from the cell body, which is the region where dendrites should be located, as confirmed by intracellular injection of Lucifer yellow [17]. As expected, wild-type motor neurons were depolarized by the amino acid applications, leading to generation of action potentials. In contrast with an even significantly larger current, the mutant motor neurons showed some depolarization but failed to generate any action potentials.

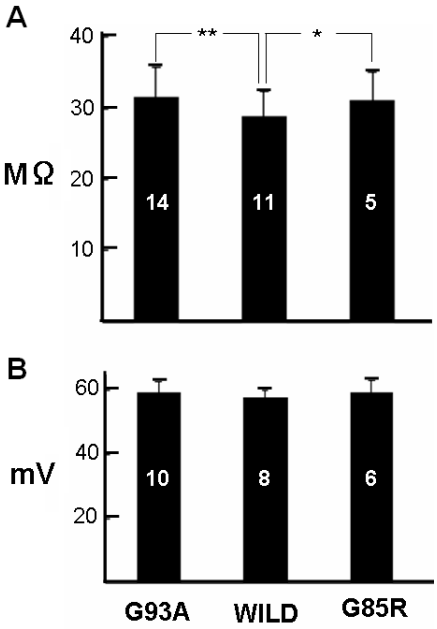

Figure 1: Histogram of **A)** membrane potential and **B)** input resistance from motor neurons from *G93A*, *G85R* and wild-type mice. Numbers in the columns indicate the number of neurons tested at around PD250. For input resistance; $p < 0.05$ for *, **.

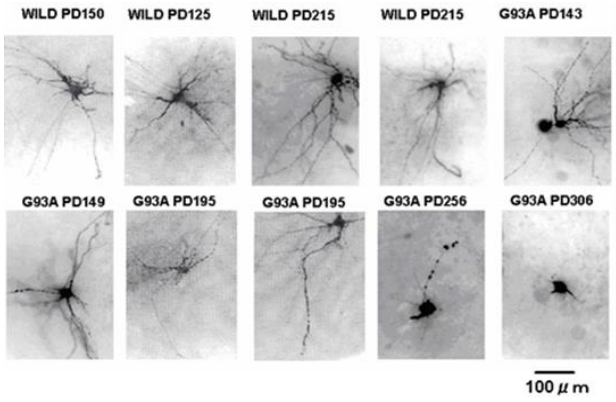

Figure 2: Morphological observation of wild-type (PD125-215) and *G93A* (PD149-306) motor neurons by Lucifer-Yellow injection.

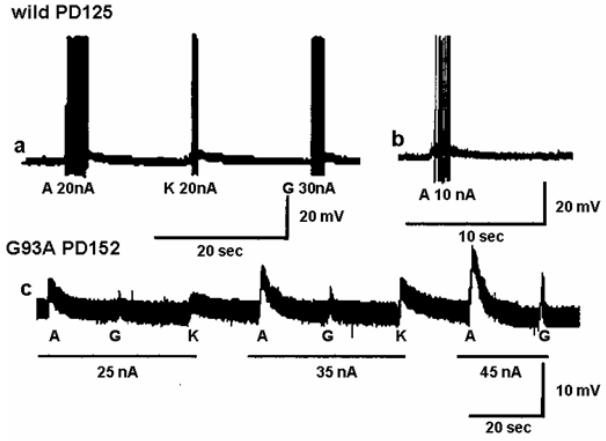

Figure 3: Intracellular recordings showing effects of ionophoretic application of the glutamate agonists, AMPA (A) and KA (K) and glutamate (G) at a site about 300 µm away from the cell body of a single motor neuron from **a & b)** a wild-type mouse at PD125 and **c)** a *G93A* mutant mouse at PD152. The applied current to release the drugs is indicated below the responses. The reaction to ionophoresis into the area where the dendrites should be located in the G93A motor neuron was very much weaker. Note that even with a much larger applied current the mutant motor neuron is not depolarized enough to generate an action potential.

Figure 4 shows a similar experiment but with bath perfusion of the excitatory amino acids onto cervical slices from wild-type and *G93A* mice. With bath perfusion, the mutant motor neurons are very responsive to AMPA and KA even at a lower concentration. These results show that the cell body still has electrical excitability and has excitatory amino acid receptors, even though the dendritic function of *SOD1* mutant mouse has been very much reduced. Figure 5 shows the results of the application of intracellular current in a motor neuron from a wild-type and mutant animal. Intracellular recordings are from the cell body, not dendrites. The amount of current necessity to evoke an action potential is much less in the mutant motor neuron than the wild-type animal. Figure 6 shows an intracellular recording from a cervical motor neuron from a *G93A* mouse at age PD195. This motor neuron shows spontaneous pacemaker-like activity which is either i) increased or decreased by ii) depolarizing or hyperpolarizing current, respectively. Spontaneous activity was only seen in a few motor neurons but is particularly interesting because in early ALS spontaneous twitching of muscles is common, reflecting the repetitive discharge of a single motor neuron with contraction of all of the muscle fibers of that motor unit.

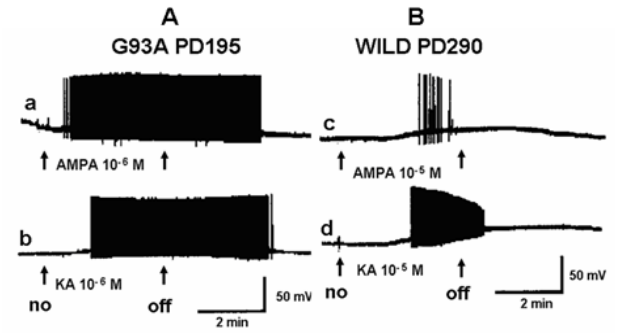

Figure 4: Intracellular recordings showing effects of bath perfusion of the glutamate agonists AMPA and KA on a motor neuron of **a & b)** a *G93A* animal (PD195) and **c & d)** a wild-type animal at PD290. Bath perfusion of **a)** AMPA (10^{-6} M). **b)** KA (10^{-6} M). **c)** AMPA (10^{-5} M). **d)** KA (10-5 M). The arrows show the on and off of the perfusion of the drug. Note that the concentrations applied to the mutant motor neuron is ten-times lower than that to the wild-type.

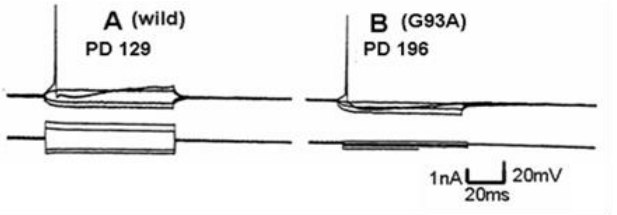

Figure 5: The difference in threshold value for excitation in response to current injection in the cell. **A)** Wild-type. **B)** *SOD1* mutant mouse. The upper trace records potential while the lower trace is applied current.

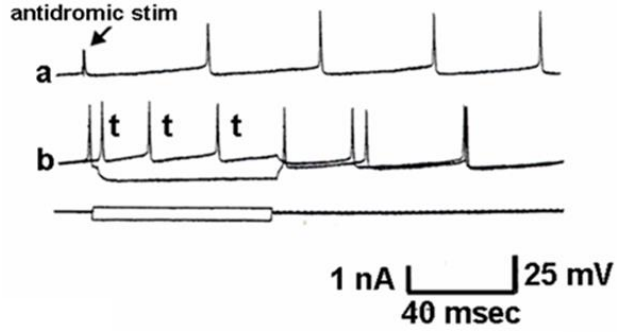

Figure 6: Typical electrical recording of a motor neuron from a *G93A* PD195 mouse showing spontaneous firing. **a)** The arrow shows the artifact of an antidromic stimulation that failed to generate a full action potential. The others are spontaneous discharges. **b)** This shows inhibition of the spontaneous discharge by hyperpolarizing current and an increase in the action potentials with the depolarization.

Figure 7 shows the effects of perfusing cervical slices with Ringer's solution containing different concentrations of calcium on the AMPA responses of wild-type and *G93A* motor neurons. The wild-type motor neuron response did not change dramatically in solutions with different calcium concentrations. However, the response of the mutant motor neuron became very unstable with higher calcium concentrations.

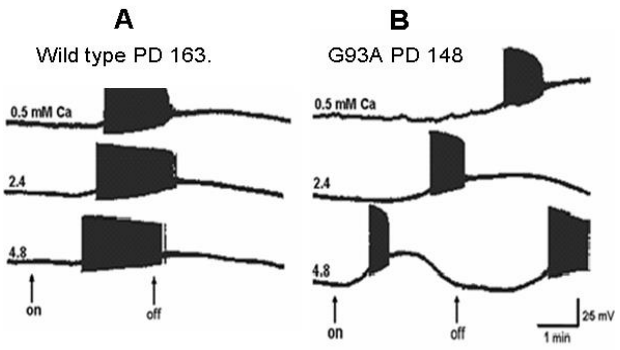

Figure 7: Influence of different calcium concentrations in the Ringer's solution on responses to perfused AMPA (10^{-5}M) in **A)** a wild-type motor neuron from an animal at PD163 and **B)** a *G93A* animal at PD148. The concentration of calcium was varied between 0.5, 2.4 and 4.8 mM. The arrows show on and off of the AMPA perfusion.

Discussion

The most striking feature we find when comparing cervical motor neurons of wild-type and *SOD1* mutant mice are the selective loss of the dendritic tree while the cell body remains active, even generating pacemaker-like action potentials. The loss of dendrites is shown dramatically in the Lucifer-Yellow filled cells. Fogarty *et al.* (2016) have previously reported the loss of synaptic spines and dendrites in *SOD1* mutant mice, but from cortical pyramidal neurons, not motor neurons [18]. The changes in electrical properties of the motor neurons are consistent with this observation. Membrane potential, always measured in the cell body, was not significantly different between wild-type and mutant motor neurons. However, membrane resistance was significantly greater in the mutants. This is what one would expect with loss of dendrites. It is also of interest that even when there has been a significant loss of dendrites, the motor neuron cell body remains electrically excitable and responsive to excitatory amino acid receptors. If anything, the cell bodies are unusually excitable, even to the point of generating spontaneous pacemaker discharges. While spontaneous pacemaker activity is common in invertebrate neurons, it has not often been observed in mammalian neurons. However, we have previously found that neurons in the medial vestibular nucleus are endogenous pacemakers and spontaneously active neurons have been recorded in spinal cord slices [19, 20]. In the case of a spontaneous motor neuron, one would expect to see contractions of the muscle fibers innervated by that motor neuron. Repetitive twitching of motor units is common in ALS patients [21].

As shown in (Figure 5), the threshold for action potential generation in the mutant motor neuron is low in comparison with the wild-type. Since the membrane potential in the mutant motor neuron is almost the same as in the wild-type, this may be a result of the difference in input resistance. Calcium, both extracellular and intracellular, plays a very important role in regulation of normal neuronal function and is a factor in neuronal damage and death [22]. This is why we studied the effects of varying extracellular calcium concentration on the responses to AMPA on wild-type and mutant mice. The response of AMPA with increasing calcium concentration in the perfused Ringer's in the wild-type cell was a gradually increased response, but an abnormal excitation was seen in the mutant cell. We hypothesize that the reason for this excessive excitation relates to intracellular calcium regulation. The mitochondria have a close relation to the endoplasmic reticulum and together, they control the concentration of intracellular calcium [23]. The rhythmical change of depolarization and hyperpolarization of the membrane potential is probably the absorption and release of the calcium between the endoplasmic reticulum and mitochondria [23]. However, further study of these effects is needed.

Conclusion

We have compared the morphologic and electrophysiologic responses of cervical motor neurons of wild-type and two mouse mutants that are good models of the human disease, ALS. The motor neurons of *SOD1* mutant mice show a progressive loss of dendrites but the cell body remains hyperexcitable, generating increased sensitivity to excitatory neurotransmitters applied to the cell body and even generating spontaneous pacemaker discharges.

Ethical Approval

This work was approved by the Animal Research Review Board of the University at Albany.

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