Postsynaptic Alteration of NR2A Subunit and Defective Autophosphorylation of alphaCaMKII at Threonine-286 Contribute to Abnormal Plasticity and Morphology of Upper Motor Neurons in Presymptomatic SOD1G93A Mice, a Murine Model for Amyotrophic Lateral Sclerosis

A. Spalloni1, N. Origlia2, C. Sgobio1, A. Trabalza1,3, M. Nutini1,4, N. Berretta1, G. Bernardi1,4, L. Domenici2,5, M Ammassari-Teule1,6 and P. Longone1

1Department of Experimental Neurology, Santa Lucia Foundation, 00143 Rome, Italy, 2CNR Institute for Neuroscience, 56124 Pisa, Italy, 3Department of Psychology, University "La Sapienza," 00185 Rome, Italy, 4Department of Neuroscience, Tor Vergata University, 00133 Rome, Italy, 5Department of Scienze e Tecnologie Biomediche, School of Medicine, University of L'Aquila, 67010, Italy and 6CNR Institute for Neuroscience, 00143 Rome, Italy

Address correspondence to Dr Patrizia Longone, Molecular Neurobiology Unit, Experimental Neurology, Santa Lucia Foundation, Via del Fosso di Fiorano, 64 00143 Rome, Italy. Email: p.longone@hsantalucia.it.

Although amyotrophic lateral sclerosis (ALS) has long been considered as a lower motor neuron (MN) disease, degeneration of upper MNs arising from a combination of mechanisms including insufficient growth factor signaling and enhanced extracellular glutamate levels is now well documented. The observation that these mechanisms are altered in presymptomatic superoxide dismutase (SOD1) mice, an ALS mouse model, suggests that defective primary motor cortex (M1) synaptic activity might precede the onset of motor disturbances. To examine this point, we assessed the composition of AMPAR and NMDAR subunits and measured the alphaCaMKII at threonine-286 in the Triton insoluble fraction from the M1 in postnatal P80-P85 SOD1G93A and wild-type mice. We show that presymptomatic SOD1G93A exhibit a selective decrease of NR2A subunit expression and of the alphaCaMKII at threonine-286/calmodulin-dependent kinase autophosphorylation at threonine-286 in the Triton insoluble fraction of upper MNs synapses. These molecular alterations are associated with synaptic plasticity defects, and a reduction in upper MN dendritic outgrowth revealing that abnormal neuronal connectivity in the M1 region precedes the onset of motor symptoms. We suggest that the progressive disruption of M1 corticocortical connections resulting from the SOD1G93A mutation might extend to adjacent regions and promote development of cognitive/dementia alterations frequently associated with ALS.

Keywords: AMPA and NMDA receptors, cortical synaptic plasticity, dendrite outgrowth, familial amyotrophic lateral sclerosis (fALS), SOD1G93A mutation, upper motor neurons

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the loss of motor neurons (MNs). Main clinical hallmarks include progressive muscle weakness, muscle atrophy, and paralysis leading to death by respiratory insufficiency (Cudkowicz et al. 2004). Familial ALS (fALS) constitutes about 5-10% of ALS cases, with mutations in the gene for the cytosolic form of the free radical-scavenging enzyme superoxide dismutase (SOD1) representing approximately 15% of fALS cases (Rosen et al. 1993; Andersen 2006).

ALS has long been considered as a lower MNs disease but degeneration of upper MNs is now well documented (Eisen and Webster 2001; Ellis et al. 2001; Maekawa et al. 2004). Neurodegenerative cortical traits consist in fragmentation of the Golgi apparatus and reduction of dendritic arbor of giant pyramidal Betz cells located within layer V of the M1 cortical region (Udaka et al. 1986). Importantly, the number of upper MNs (Gredal et al. 2000) as well as their perikarya and nuclear volumes are unaltered in ALS patients suggesting that dendrite morphology and synaptic function of upper MNs are selectively affected. Similar to ALS patients, SOD1G93A mice, a murine model of fALS, show neuronal degeneration in cortical motor and extramotor regions (Leichsenring et al. 2006) which is detected prior to the onset of clinical symptoms. Vulnerability of MNs in ALS likely arises from a combination of several mechanisms including protein misfolding, mitochondrial dysfunction, oxidative damage, defective axonal transport, inflammation, insufficient growth factor signaling, and enhanced extracellular glutamate levels inducing neuronal excitotoxicity (Shaw and Ince 1997; Plaitakis et al. 1988; Spreux-Varoquaux et al. 2002; Vucic et al. 2008; Rothstein 2009).

Glutamate, the major excitatory neurotransmitter in the central nervous system, binds to ionotropic and metabotropic receptors. There is evidence that activation of the ionotropic glutamate receptors, NMDARs, triggers signal transduction cascades that control neuronal survival and activity-dependent synapse modifications (Furukawa et al. 2005). Moreover, NMDAR subunit expression is downregulated in response to excessive activation of this receptor in vitro and in vivo models of excitotoxicity (Gascon et al. 2005). Based on these observations, we reasoned that the enhancement of extracellular fluid glutamate levels present in presymptomatic SOD1G93A mice (Guo et al. 2000) could modify the pattern of glutamate receptors subunit expression at upper MNs synapses affecting, in turn, M1 synaptic plasticity and neuronal morphology. To explore this possibility, we assessed the composition of AMPAR and NMDAR subunits and measured alphaCaMKII at threonine-286/alphaCaMKII autophosphorylation at threonine-286 in the Triton insoluble fraction (TIF) from the M1 neurons in SOD1G93A and wild-type mice. Synaptic efficacy at upper MNs synapses and upper MNs morphology were then evaluated by measuring synaptic plasticity and dendritic arbor in Golgi-stained pyramidal neurons in the M1 region. Here, we show a significant decrease in NR2A subunit expression and alphaCaMKII autophosphorylation at threonine-286 in the TIF of SOD1G93A.
M1 synapses. Consistent with this finding, alterations in synaptic plasticity and a reduction in dendritic outgrowth were detected in M1 neurons of mutant mice prior to the onset of motor symptoms.

Materials and Methods

Transgenic Mice

B6SJL-TgN (SOD1-G93A) 1Gur mice expressing the human G93A Cu/Zn SOD1 mutation were from our own colony, bred at the Santa Lucia Foundation animal facility, and originally obtained from the Jackson Laboratories (Bar Harbor). Mice hemizygous for the mutated SOD1 (SOD1G93A) human gene and control littermates (WT) were housed in groups of 4 in a temperature controlled room (22 °C) with a light:dark 12:12 cycle (lights on 7:00–19:00 h). Food and water were given ad libitum. Screening for the presence of the human transgene was performed on tail tips homogenized in phosphate buffer saline, freeze/thawed twice, and centrifuged at 14 000 rpm for 15 min at 4 °C. The P2 and TIFs were used for the western blot analysis. The same amount of protein from P2 and TIF (15 μg) was applied to SDS-PAGE and electroblotted to polyvinylidene difluoride membranes. Western blots were performed as described (Spalloni et al. 2006). Briefly, after an overnight incubation with the antibody to be tested, immunoreactivity was detected using Horseradish Peroxidase-coupled anti-IgG antibody, visualized with the ECLplus enhance chemiluminescence kit (Amersham Biosciences) and analyzed with the Storm Scanner Control and the proprietary Image-Quant TL software (Amersham Biosciences). Blots were developed to be linear in the range used for densitometry as determined with preliminary results. The complete list of the antibodies used in the study with the working dilutions and the supplier is presented in the Supplementary Table 1. For each antibody, preliminary experiments were performed to obtain a linear standard curve, and all the quantification have been carried out within each antibody linear range and normalized for the beta-actin levels in the same membrane. For the quantification of the phosphorylated alphaCaMKII (pCaMKII), the optical density (OD) of the pCaMKII and alphaCaMKII bands were normalized to their respective beta-actin controls on the same blot. The normalized pCaMKII intensity was then measured over the normalized alphaCaMKII intensity. The final calculations were performed as described in Ng et al. (2010).

Preparation of Protein Extracts and Characterization of Subcellular Compartments

The M1 region (Fig. 1A) was dissected using a 1 mm coronal mouse brain slicer (Zivic Miller) and immediately processed. M1 tissues (WT n = 6 and SOD1G93A n = 6, unless otherwise indicated) were homogenized with a glass-teflon homogenizer in ice-chilled homogenization buffer (4 mM Hepes pH 7.4 containing 320 mM sucrose, 2 mM ethylenediaminetetraacetic acid) with a complete set of protease inhibitors cocktail (Sigma) as described in Gardoni et al. (2006) with some modifications (see Fig. 1B). The homogenate was centrifuged at 710 × g for 10 min to remove nuclei and debris (nuclei-associated membranes, P1). The low-speed supernatant (S1) was centrifuged at 13 000 × g for 20 min to obtain the crude membranes fraction (P2), which was resuspended in homogenization buffer, and the S2 fraction (cytosol). To half of the P2 fraction, a 1% final Triton-X100 was added then this mixture was slowly rocked at 4 °C for 15 min and centrifuged at 200 000 × g for 2 h to obtain the pellet (P3), referred as the TIF and the supernatant (S3) referred as the Triton-soluble fraction (TSF). The TIF was then resuspended in homogenization buffer with protease inhibitor. Total protein content was measured in all the fractions with the Bio-Rad Protein Assay (Bio-Rad). The primary visual cortex (V1, approximately plates 55–60, of the atlas of Franklin and Paxinos 2008) was dissected and processed as described for the M1.

Western Blots

The P2 and TIFs were used for the western blot analysis. The same amount of protein from P2 and TIF (15 μg) was applied to SDS-PAGE and electroblotted to polyvinylidene difluoride membranes. Western blots were performed as described (Spalloni et al. 2006). Briefly, after an overnight incubation with the antibody to be tested, immunoreactivity was detected using Horseradish Peroxidase-coupled anti-IgG antibody, visualized with the ECLplus enhance chemiluminescence kit (Amersham Biosciences) and analyzed with the Storm Scanner Control and the proprietary Image-Quant TL software (Amersham Biosciences). Blots were developed to be linear in the range used for densitometry as determined with preliminary results. The complete list of the antibodies used in the study with the working dilutions and the supplier is presented in the Supplementary Table 1. For each antibody, preliminary experiments were performed to obtain a linear standard curve, and all the quantification have been carried out within each antibody linear range and normalized for the beta-actin levels in the same membrane. For the quantification of the phosphorylated alphaCaMKII (pCaMKII), the optical density (OD) of the pCaMKII and alphaCaMKII bands were normalized to their respective beta-actin controls on the same blot. The normalized pCaMKII intensity was then measured over the normalized alphaCaMKII intensity. The final calculations were performed as described in Ng et al. (2010).

Figure 1. The primary (M1) motor cortex subcellular fractionation. Representative cresyl violet section of the M1 motor cortex region (bottom A, scale bar; 200 μm), also shown (gray) in the drawing extracted from the mouse atlas of Franklin and Paxinos (2008, top A). Description of the biochemical fractionation method used in the present study (B). The M1 region isolated fractions were separated by SDS-PAGE, and the blots were probed with antibodies against synaptophysin, NMDAR1, NR2A, PSD-95, and GluR2 (C). P2, crude membrane fraction; TIF, P1, nuclei-associated membranes; S2, cytosol fraction; S3, triton-soluble fraction. The sign (−) indicates the WT-loaded lines; the sign (+) indicates the SOD1G93A-loaded lines. M1: primary motor cortex, M2: secondary motor cortex, cg: cingulum, CPu: caudate putamen.
Genotypic difference in expression protein levels was estimated by one-way analysis of variance (ANOVA). Differences were considered significant for $P < 0.05$.

**Immunostaining for Confocal Microscopy**
Mice (WT $n = 3$ and SOD1G93A $n = 3$) were deeply anesthetized (chloral hydrate, 400 mg/kg) via intraperitoneal injection and perfused through the left ventricle with 0.9% NaCl followed by 4% paraformaldehyde (PAF) in phosphate buffer (PB, 0.1 M pH 7.4). The brains were then removed and postfixed in PAF. Free floating sections (30 µm) were permeabilized with 0.3% TritonX-100/4% donkey serum in PB and then incubated with the primary antibodies in different combinations: monoclonal NeuN (CHEMICON; 1:100), monoclonal SMI32 (Sternberg Monoclonal Inc.; 1:1000), polyclonal Glial Fibrillary Acidic Protein (GFAP) (CHEMICON; 1:1000), for 24—72 h at 4°C and then with the appropriate secondary antibodies. Sections were mounted, air-dried, and coverslipped, images were acquired with a confocal laser scanning microscope (LEICA Sp5).

**Electrophysiological Recordings**
Mice were deeply anesthetized using urethane (20% solution, 0.1 mL/100 g body weight) via intraperitoneal injection and then decapitated after disappearance of the tail pinch reflex. The brains were rapidly removed, and thick coronal sections (400 µm) containing the M1 region (1—2 mm anterior to the bregma) were made on a vibratome. All steps were performed in ice-cold artificial cerebrospinal fluid solution (ACSF) (in mM: NaCl, 119; KCl, 2.5; CaCl2, 2; MgSO4, 1.2; NaH2PO4, 1; NaHCO3, 26.2; glucose, 11) bubbled with 95% O2/5% CO2. Prior to recording, slices were maintained for at least 1 h in a recovery chamber containing oxygenated ACSF solution at room temperature. During electrophysiological recordings, slices were perfused at a rate of 2.5—3 mL/min with oxygenated ACSF, at 33 ± 1°C. Extracellular field potentials (FPs) were recorded in layer II/III of the M1 region using pulled glass capillaries; the stimulating concentric bipolar electrode was placed in the same layer (medially to the recording pipette). The amplitude of the FPs was used as a measure of the evoked population excitatory synaptic response (Aroniadou and Keller 1995). Amplitudes of FPs were calculated at different stimulus intensities to obtain input-output curves. Experiments to assess paired-pulse (PP) stimulation were performed as well; 2 consecutive stimuli were applied at different interstimulus intervals (ISIs, 25—200 ms). The stimulation intensity was set to a value yielding 50—60% of maximal amplitude in the first pulse, and the response to this first pulse remained constant during the whole PP protocol. PP-induced percentage change was calculated as the ratio of the peak amplitude of the second FP to the first one. Baseline responses were obtained with a stimulation intensity that yielded 50—60% of maximal amplitude. FP amplitude was monitored every 20 s and averaged every 3 responses by online data acquisition software (Anderson and Collingridge 2001). After 10 min of stable baseline recording, repeated theta burst stimulation (4 Tris-buffered saline [TBS] at 10-s interval, each consisting of 10 bursts of 5 pulses at 100 Hz, separated by 200 ms) or low-frequency stimulation (LFS; 1800 pulses at 2 Hz) was used to induce long term potentiation (LTP) or long term depression (LTD), respectively. LTP and LTD magnitude was quantified as percentage of the mean FP amplitude (± standard error of the mean) between the 40th and the 50th min after induction protocol compared with control. Statistical comparison between FP amplitudes measured during baseline and during the last 10 min of recording following TBS or LFS was performed by applying a 2-way repeated measure ANOVA.
followed by Holm-Sidak all-pairwise multiple comparison procedure. Differences were considered significant for \( P < 0.05 \).

**Golgi-Cox Impregnation of Brain Tissue**

Mice (WT \( n = 5 \); SOD1G93A \( n = 5 \)) were anesthetized and perfused as above. The brains were dissected, immersed in a standard Golgi-Cox solution (1% potassium dichromate/1% mercuric chloride/0.8% potassium chromate) according to the method described by Glaeser and Van der Loos (1981), and stored at room temperature for 6 days. They were transferred to a sucrose solution (30%) for 5 days and then sectioned coronally (150 μm) using a vibratome. Sections were mounted on gelatinized slides, stained according to the Gibb and Kolb (1998) method, and covered with Permount.

**Measurement of Dendrite Branching**

Five brains from each genotype were processed for morphological analyses. Measurements were performed on impregnated neurons identified under low magnification (20x/0.5NA). Within each hemisphere, 3 pyramidal cortex neurons with the soma in layer V and apical dendrites reaching layers III and IV were selected in the M1 region (Bregma 1.98–1.78 mm, Franklin and Paxinos 2008, see Fig. 1A) and subsequently analyzed under higher magnification (63x/0.75NA). Only fully impregnated pyramidal neurons displaying dendritic trees without obvious truncations and isolated from neighboring impregnated neurons were retained for the analysis (Vyas et al. 2002). Morphological measurements were made by an experimenter blind to the genotype of the animal. Because no interhemispheric difference was detected, the data were pooled so that 6 neurons per animal were considered in each analysis. Measurements were carried out using a microscope (DMLB, Leica) equipped with a motorized stage and a camera connected to a software for morphological analyses allowing quantitative 3D analysis of complete dendritic arborization (Neurolucida 7.5; MicroBrightField, Inc.). The length, diameter, and the branch nodes of the dendritic trees were quantified tracing the entire apical and basal dendrite arbor, then performing Sholl analyses. The basal dendritic trees were required to be well discernible from neighboring impregnated dendrites, to have no breaks in staining, and at least one 4-order branch to maximize the possibility of counting spines until the most distal branch order present in this genotype. Briefly, using the center of the soma as reference point, dendritic length, diameter, and branch points were measured as a function of their radial distance from the soma by adding up all values in each successive concentric segment (segment radius: 25 μm). Differences in dendrite length, diameter, and branch points were measured as a function of their radial distance from the soma by adding up all values in each successive concentric segment (segment radius: 25 μm). Differences were considered significant for \( P < 0.05 \).

**Results**

**Characterization of Subcellular Compartments**

To evaluate the effectiveness of the fractionation method (Fig. 1B) and the purity of the subcellular fractions obtained from the M1 (Fig. 1A) tissues, we have analyzed by western blotting each compartment with specific protein markers (see Fig. 1C). The synaptic vesicle membrane protein synaptophysin, enriched in the presynaptic terminals was concentrated in the nuclei-associated membranes (P1), in the crude membrane fraction (P2) and in the Triton-soluble fraction (S3). The NMDAR subunits NR1 and NR2A and the AMPAR subunit GluR2 were found enriched in the P2 fraction, in the TIF and in P1, but not detectable in the S2 and S3 fractions. The postsynaptic density (PSD) protein PSD-95 was found in the P2, TIF, and P1 fractions and not detectable in the S2 and S3 fractions. Confocal immunofluorescence analyses performed in the M1 region at P85 revealed no neuronal (NeuN and SMI32) or glial (GFAP) difference between WT (Fig. 2A–G) and SOD1G93A mice (Fig. 2D–H).

**SOD1G93A Mice Show Reduced Content of NR2A Subunit and Altered alphaCaMKII Phosphorylation in the TIF of M1 Neurons Synapses**

Increased glutamate levels are a seminal feature of the ALS human patients and of the mouse models as well. Glutamate acts on the ionotropic and metabotropic receptors, key mediators of the excitatory synaptic transmission in the brain. The ionotropic glutamate receptor AMPARs are required for spine stabilization and maintenance, whereas the NMDARs have been implicated in the regulation of dendritic spine morphology, specifically in the formation of new spines and filopodia (for review, see Ethell and Pasqueau 2005). While the metabotropic glutamate receptors, particularly the mGluR5 subunit, regulates the glutamate-dependent development of the mouse cerebral cortex (Hannan et al. 2001; Wijetunge et al. 2008; Tsanov and Manahan-Vaughan 2009). In addition, all of them contribute to the induction and maintenance of synaptic plasticity (Newpher and Ehlers 2009).

When we examined the protein levels of the GluR1 and GluR2 AMPAR subunits in the P2 and in the TIF prepared from the M1 region of SOD1G93A mice and WT littermates, we found
Motor Cortex Alterations in Presymptomatic SOD1<sup>G93A</sup> Mice

Emanuela Spalloni, Valeria Marzolo, Paola Rossi, Maria D. Fabiani, Emilio Lidow, and Roche C. Kass

Cortical Synaptic Plasticity Is Altered in SOD1<sup>G93A</sup> Mutants

The functional consequences of the upper MNs molecular abnormalities in the SOD1<sup>G93A</sup> mice were estimated by measuring basal neurotransmission and plasticity at upper MNs synapses. We first examined synaptic transmission by estimating input–output curves which depict the relationship between the stimulus intensity and the amplitude of FPs. We observed that maximal FPs amplitude did not differ between control and SOD1<sup>G93A</sup> mice (0.99 ± 0.09 mV and 0.96 ± 0.06 mV, respectively, \(P = 0.785\)) and that the input–output curves in control (\(n = 10\) slices, 6 mice) and SOD1<sup>G93A</sup> (\(n = 11\) slices, 6 mice) overlapped (Fig. 5A). Furthermore, we investigated PP facilitation in layer II/III of M1 using PP stimulation. Specifically, we analyzed the amplitude of the responses induced by pairs of stimuli delivered at different ISIs (25–200 ms) by measuring the ratio between the second and the first response amplitudes (PP ratio). The PP ratio is expressed as percentage change of the first pulse respective to the first one; this ratio is calculated in the presence of a stable baseline with an amplitude of the first response. As shown in Figure 5B, PP

![Figure 4](https://academic.oup.com/cercor/article-abstract/21/4/796/287109)

**Figure 4.** SOD1<sup>G93A</sup> expression decreases alphaCAMKII phosphorylation at threonine-286 in the TIF of the M1 region. The TIF from WT and SOD1<sup>G93A</sup> mice were analyzed by western blot with PSD-95, SAP97, alphaCaMKII, betaCaMKII, and pCaMKIIalpha antibodies. The histograms show the quantification of PSD-95 and SAP97 (A), alphaCaMKII and betaCaMKII (B), and the phosphorylated form of alphaCaMKII at threonine-286 (pCaMKIIalpha, C). Data, presented as a ratio (X100), are normalized to the beta-actin and plotted to the expression in the WT littermates. Data are presented as mean ± standard error of the mean, * \(P < 0.05\) by one-way ANOVA. The sign (±) indicates the WT-loaded lines; the sign (+) indicates the SOD1<sup>G93A</sup>-loaded lines.
Mice overexpressing the SOD1G93A gene show molecular abnormalities in the TIF of M1 cortical synapses including a specific reduction of NR2A subunit expression and a decrease of the alphaCaMKII autophosphorylation at threonine-286. These abnormalities occur prior to the onset of motor symptoms and are accompanied by robust alterations in upper MNs synaptic plasticity and morphology. The observation that the NR1 and NR2B subunits and the AMPAR GluR1 and GluR2 potentiation of FPs in WT (144 ± 10% of control, n = 8 slices, 4 mice, Fig. 5C) and SOD1G93A slices (134 ± 9% of control, n = 8 slices, 4 mice) with no group difference in the mean LTP (P = 0.709). Notably, the second TBS induced a further potentiation of FPs in WT slices (194 ± 12% of control before the first TBS, P < 0.001) but did not SOD1G93A slices (125 ± 6% of control before the first TBS Fig. 5C, P = 0.239). Thus, in SOD1G93A slices, LTD saturation is achieved after the first TBS, while in controls slices, LTD is saturated after a second TBS stimulation since a third TBS failed to produce a further significant increase of LTD magnitude in WT slices (see Supplementary Fig. 3). LTD was then compared across genotypes using an LFS protocol that was previously shown to reliably induce an NMDA-dependent form of LTD in the motor cortex (Castro-Alamancos et al. 1995; Hess and Donoghue 1996; Rioult-Pedotti et al. 2000). In Figure 5D, we reported that LFS induced a slight but significant depression of FP amplitude in WT slices (85 ± 3% of control, n = 6 slices, 3 mice, P < 0.05 vs. baseline recordings) which could be reverted by TBS applied 40 min after its induction (116 ± 9% of control before LFS, P < 0.05 vs. average amplitude after LFS). In contrast, LTD in SOD1G93A slices was strongly enhanced compared with WT slices (52 ± 7%, n = 6 slices, 3 mice; P < 0.05 vs. WT after LFS) and could not be reverted by TBS applied at the same time point (51 ± 7% of control before LFS).

Dendrite Arborization of M1 Cortical Neurons Is Reduced in SOD1G93A Mice

Figure 6 shows Golgi-stained pyramidal M1 neurons (Fig. 6A, B top) and corresponding neurolucida drawings (Fig. 6A, B bottom) in WT and SOD1G93A mice and the Sholl analysis data for dendrite length (Fig. 6C) and branch nodes (Fig. 6D) on apical and basal dendrites. Statistical analyses performed on these variables in the apical dendrite compartment did not reveal any difference between genotypes. Conversely, in the basal dendrite compartment, a main effect of genotype was found for dendrite length (F1,6 = 13.03, P < 0.05) and branch nodes (F1,6 = 9.49, P < 0.05). In addition, a significant “genotype × radial distance from the soma” interactions (dendrite length, F1,7,6 = 8.01, P < 0.05; branch nodes F1,6,6 = 2.46, P < 0.05) revealed that morphological measurements performed at increasing distance from the soma evolved differently according to the genotype. Post hoc comparisons then showed that dendrites laying 50-100 μm from the soma were shorter, and those laying 25-50 μm from the soma had fewer nodes in SOD1G93A than in WT mice. No genotypic difference in dendrite diameter was found in any compartment (apical, WT: 1.01 ± 0.04 μm, SOD1G93A: 1.38 ± 0.05 μm, F1,6 = 2.44, P > 0.05); basal, WT: 1.01 ± 0.04 μm, SOD1G93A: 0.93 ± 0.03 μm, F1,6 = 3.76, P > 0.05).

Discussion

Mice overexpressing the SOD1G93A gene show molecular abnormalities in the TIF of M1 cortical synapses including a specific reduction of NR2A subunit expression and a decrease of the alphaCaMKII autophosphorylation at threonine-286. These abnormalities occur prior to the onset of motor symptoms and are accompanied by robust alterations in upper MNs synaptic plasticity and morphology. The observation that the NR1 and NR2B subunits and the AMPAR GluR1 and GluR2
are normally expressed at the same postsynaptic site suggests that a compartment-specific alteration of NR2A expression and a decrease in the mechanisms activating constitutive alpha-CaMKII both contribute in triggering functional and morphological defects in SOD1G93A motor cortex circuits.

NMDARs include the structural NR1 subunits and 4 NR2 subunits (NR2A–D), but in the brain, NR2A and NR2B are the most expressed ones (Watanabe et al. 1993; Monyer et al. 1994; Sheng et al. 1994). In the mature brain, NR2A are preferentially located on synaptic terminals, while NR2B are mostly extrasynaptic (Stocca and Vicini 1998; Townsend et al. 2003). There is evidence that forebrain and cortical NR2A/NR2B ratio is developmentally regulated, with NR2A partly replacing NR2B during ontogenesis (Flint et al. 1997; Mierau et al. 2004). In adult rat brains, NR2A and NR2B control different aspects of cortical synaptic plasticity. It is well accepted that LTP and LTD, as regarded by their threshold of induction and saturation, are influenced by the NR2A/NR2B expression and ratio, with a stronger contribution of NR2B to LTD induction, as opposed to NR2A more involved in LTP (Massey et al. 2004; Miwa et al. 2008; Yashiro and Philpot 2008) although the actual role of each subunit in mediating the 2 forms of synaptic plasticity is still controversial (Barria and Malinow 2005; de Marchena et al. 2008). Conversely, it is well known that NMDARs participate in LTP and LTD maintenance via a number of signaling molecules, among which the abundantly expressed synaptic protein alphaCaMKII (Cammarota et al. 2002; Merrill et al. 2005). This protein preferentially binds the NR2B subunit (Strack and Colbran 1998; Leonard et al. 1999; Strack et al. 2000). However, although alphaCaMKII is activated in the presence of Ca²⁺, there is evidence that only autophosphorylation at threonine-286 allows it to remain activated in a Ca²⁺
independent fashion. Notably, the generation of mice with a targeted point mutation preventing autophosphorylation at threonine-286 (alphaCaMKII T286A knock-in mice, Giese et al. 1998) has revealed the importance of this mechanism for cortical synaptic plasticity. In particular, the observation that alphaCaMKII T286A knock-in mice fail to show cortical LTP in a variety of protocols including TBS, spike pairing, and postsynaptic depolarization paired with low-frequency presynaptic stimulation (Hardingham et al. 2003) points to autophosphorylation alphaCaMKII at threonine-286 as a critical molecular switch for LTP induction/maintenance. Thus, the concomitant reduction of NR2A expression and alphaCaMKII autophosphorylation at threonine-286 in the TIF of SOD1G93A M1 neurons delineates a postsynaptic pattern of NMDARs-signaling abnormalities which might be an integral component of 1) the reduced susceptibility to synaptic potentiation, as indicated by the saturation of LTP by a single TBS and 2) the resistance to potentiation of previously depressed synapses in presymptomatic SOD1G93A mutants. Hence, the enhanced LTD expression found in the same mice could be a secondary effect of the reduced propensity to potentiation. Indeed, such an unbalance toward depression of synaptic efficacy might be driven by the modification of the NR2A/NR2B ratio in favor of NR2B.

NMDARs composition also plays a crucial role in neuronal structural development. For example, the prominent role of the NR2A subunit in branch clustering has been also confirmed by experiments in which both exogenous subunit expression and endogenous subunit knockdown were used to shift synaptic NMDARs composition (Ewald et al. 2008). In particular, these authors showed that the knocking down of any subunit produces a retraction of dendritic arbor development whereas the selective knocking down of NR2A specifically disrupted branch clustering. These observations therefore support the view that the signal through NR2A is crucial for the formation of higher dendritic branch orders. In agreement with this, we observed a strong reduction of dendrite branching in SOD1G93A M1 neurons in which postsynaptic expression of NR2A is decreased. Our findings therefore agree with a scenario in which an altered NR2A expression decreases dendritic branch complexity and reduces LTP at cortical synapses, as already shown in the hippocampus both in vitro (Liu et al. 2004; Massey et al. 2004) and in vivo (Fox et al. 2006). Indeed, a direct implication of CaMKII signaling in Ca2+-dependent development of dendritic arbor in cortical neurons has also been demonstrated although other members of the CaMK family might also be involved (Wayman et al. 2008).

One point to be considered is that we failed to find substantial histopathological lesions in presymptomatic SOD1 mice at the 3-month time point. For example, using the neuron-specific nuclear protein NeuN (Inda et al. 2007) and controlling the nonphosphorylated neurofilament marker SM132 expression (Spalloni et al. 2004; Avossa et al. 2006; Thangavel et al. 2009), we did not observe any MN loss in the M1 region contrary to the early report by Zang and Cheema (2002), but in agreement with the more recent one by Kassa et al. (2009). Similarly, estimation of gliosis by means of GFAP immunostaining revealed comparable levels in mutant and WT mice, with only occasionally activated astrocytes, as already documented (Leichsenring et al. 2006). These observations therefore confirm that upregulation of inflammatory genes is a peculiarity of terminal SOD1 mutants (Kassa et al. 2009). Surprisingly, few studies on changes in cortical neuronal connectivity have been conducted in relation to ALS despite one case study, based on postmortem analysis of cortical morphology in a 38-year-old man with MN disease and dementia. This study revealed a reduction in the frontal and temporal lobes dendritic arbor accompanied by the presence of proximal dendritic varicosities and truncated dendrites (Ferrer et al. 1991). In presymptomatic SOD1G93A mutants, we reported a dramatic reduction of the dendritic arbor in the prelimbic/infralimbic medial prefrontal regions accompanied by a behavioral deficit in tasks assessing prefrontal-dependent executive functions (Sgobio et al. 2008). Importantly, the observation that no change occurred in the primary visual cortex area indicated that defective dendritic outgrowth in SOD1G93A M1 cortex might be region selective (Sgobio 2006). In line with the view that SOD1 mutations interfere with MN connectivity, postnatal examination of lumbar MNs of SOD1 mice carrying the G85R mutation revealed abnormal dendrite arborization and reduced excitability (Amendola and Durand 2008). Moreover, studies focusing on TDP-43, an evolutionarily conserved RNA-binding protein implicated in the pathogenesis of sporadic and fALS, have shown that the expression of human TDP-43 (hTDP-43) in Drosophila-cultured neurons promoted dendritic branching while this function was attenuated by mutations associated with ALS (Lu et al. 2009).

Collectively, these data suggest that abnormal synaptic plasticity and connectivity of SOD1G93A upper MNs might have adverse effects on widespread cortical networks and their dynamics. For example, there is evidence that formation of dendritic arbors is an activity-dependent regulated process since its size and geometry are adjusted as a function of the level and the distribution of inputs received (Tripathi et al. 2008). Based on these data, it is therefore tempting to speculate that defective synaptic plasticity and neuronal connectivity initially localized in the M1 region might trigger a progressive disruption of corticocortical connections in adjacent frontal regions governing cognitive and emotional functions. This mechanism, which might provide some support to the intriguing correlation existing between motor and executive function deficits in ALS, would therefore contribute to the development of cognitive/dementia impairments frequently observed in ALS patients (Strong et al. 1996; Grossman et al. 2008; Raaphorst et al. 2010).

Supplementary Material
Supplementary material can be found at: http://www.cerebro.org/

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References


