Corticospinal Motor Neurons and Related Subcerebral Projection Neurons Undergo Early and Specific Neurodegeneration in \(hSOD1^{G93A}\) Transgenic ALS Mice

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Corticospinal Motor Neurons and Related Subcerebral Projection Neurons Undergo Early and Specific Neurodegeneration in hSOD1<sup>G93A</sup> Transgenic ALS Mice

P. Hande Özdinler, 1,2,3 Susanna Benn, 4 Ted H. Yamamoto, 1,2,3 Mine Güzel, 1,2,3 Robert H. Brown Jr, 4 and Jeffrey D. Macklis 1,2,3

1MGH-HMS Center for Nervous System Repair, Departments of Neurosurgery and Neurology, and Program in Neuroscience, Harvard Medical School, 2Nayef Al-Rodhan Laboratories, Massachusetts General Hospital, and 3Department of Stem Cell and Regenerative Biology and Harvard Stem Cell Institute, Harvard University, Boston, Massachusetts 02114, and 4Department of Neurology, MassGeneral Institute for Neurodegenerative Diseases, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts 02129

Amyotrophic lateral sclerosis (ALS) is characterized by predominant vulnerability and central degeneration of both corticospinal/corticobulbar motor neurons (CSMN; “upper motor neurons”) in cerebral cortex, and spinal/bulbar motor neurons (SMN; “lower motor neurons”) in spinal cord and brainstem. Increasing evidence indicates broader cerebral cortex pathology in cognitive, sensory, and association systems in select cases. It remains unclear whether widely accepted transgenic ALS models, in particular hSOD1<sup>G93A</sup> mice, undergo degeneration of CSMN and molecularly/developmentally closely related populations of nonmotor projection neurons [e.g., other subcerebral projection neurons (SCPN)], and whether potential CSMN/SCPN degeneration is specific and early. This relative lack of knowledge regarding upper motor neuron pathology in these ALS model mice has hindered both molecular-pathophysiologic understanding of ALS and their use toward potential CSMN therapeutic approaches. Here, using a combination of anatomic, cellular, transgenic labeling, and newly available neuronal subtype-specific molecular analyses, we identify that CSMN and related nonmotor SCPN specifically and progressively degenerate in hSOD1<sup>G93A</sup> mice. Degeneration starts quite early and presymptomatically, by postnatal day 30. Other neocortical layers, cortical interneurons, and other projection neuron populations, even within layer V, are not similarly affected. Nonneuronal pathology in neocortex (activated astroglia and microglia) is consistent with findings in human ALS cortex and in affected mouse and human spinal cord. These results indicate previously unknown neuron type-specific vulnerability of CSMN/sensory and association SCPN, and identify that characteristic dual CSMN and SMN degeneration is conserved in hSOD1<sup>G93A</sup> mice. These results provide a foundation for detailed investigation of CSMN/SCPN vulnerability and toward potential CSMN therapeutics in ALS.

Introduction

Independent, but presumed to be mechanistically related, degeneration of both “upper” [corticospinal/corticobulbar motor neurons (CSMN)] and “lower” [spinal/bulbar motor neurons (SMN)] components of motor neuron circuitry (Lefebvre et al., 1998; Gavrilina et al., 2008) distinguishes amyotrophic lateral sclerosis (ALS) from disorders characterized by marked vulnera-

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R. H. Brown Jr’s present address: Department of Neurology, Northwestern University, Feinberg School of Medicine, Chicago IL 60611.

Correspondence should be addressed to Jeffrey D. Macklis at the above address, E-mail: jeffrey_macklis@hms.harvard.edu; or P. Hande Özdinler at her present address, E-mail: ozdinler@northwestern.edu.

Both inherited (familial) and sporadic forms of ALS show similar cardinal features: clinical course and neuropathology (Bendotti and Carri, 2004; Boillée and Cleveland, 2004; Bruijn et al., 2004; Pasinelli and Brown, 2006). Thus, transgenic mouse models generated based on genetics of even relatively rare inherited forms of ALS offer important tools with which to investigate both sporadic and familial ALS, if patient pathology is faithfully reflected (Gurney, 1997, 2000). Toxic “gain of function” mutation in the superoxide dismutase-1 (SOD1) gene, linked to ~10–
20% of familial ALS (~1–2–5% of all cases) is known to result in an ALS-like SMN degeneration phenotype in hSOD1G93A transgenic mice (Gurney et al., 1994; Tu et al., 1996; Gurney, 1997; Kunst et al., 1997; Beckman et al., 2001; Wong et al., 2002; Bendoroti and Carri, 2004; Wengenack et al., 2004; Hegedus et al., 2007). In contrast to considerable investigation of SMN in hSOD1G93A and other models (Chiu et al., 1995), much less is known about cortical components (degeneration of SMN and subcerebral projection neurons (SCPN) that constitute cognitive, association, and integrative sensory circuitry (Zang and Cheema, 2002; Lobisger et al., 2005; Yamanaka et al., 2006), and whether widely distributed nonneuronal pathology observed in select ALS patients is recapitulated in hSOD1G93A mice. Reports on hSOD1G93A mice have suggested that their neuropathology is restricted mainly to spinal and bulbar motor neuron degeneration, but not in cortex (Wong et al., 2002; Ralph et al., 2005; Niessen et al., 2006), potentially suggesting that hSOD1G93A mice do not represent the complete human ALS phenotype. The lack of data on CSMN is perhaps not surprising, since only ~6000 SMN exist per hemisphere in mice, intersected with millions of other cortical pyramidal neurons in the same region and layer V of motor cortex. Identification of specific degeneration of CSMN and related SCPN is enabled with recently identified molecular markers and knowledge about CSMN/SCPN developmental relationship (Gurney, 2000; Arlotta et al., 2005; Molyneaux et al., 2005, 2007, 2009; Tomassy et al., 2010). A few studies report corticospinal tract involvement in hSOD1G93A mice (Zang and Cheema, 2002; Lobisger et al., 2005; Yamanaka et al., 2006), although complexities noted above limited analyses. One study, using retrograde labeling from spinal cord at postnatal day 60 (P60), reported CSMN loss (Zang and Cheema, 2002), although it was difficult to assess the following: (1) whether observed reduction in retrogradely labeled neurons was due to abnormal retrograde axonal transport or true neuronal loss; (2) whether specific CSMN degeneration or broader cortical degeneration occurs; (3) whether observed abnormalities develop before P60; or (4) whether broader populations of SCPN/other neurons are affected.

To provide a foundation for increasingly detailed investigation of CSMN/SCPN biology in ALS and for critical analysis of cellular and molecular mechanisms of shared biology between CSMN/SCPN and SMN components of motor neuron circuitry, we investigated whether distinct and specific degeneration of CSMN (with additional but not yet clarified non-CSMN cortical degeneration), as observed in ALS patients (Rösler et al., 2000; Zanette et al., 2002; Stewart et al., 2006), is faithfully reproduced in hSOD1G93A mice. Our results allow increasingly detailed cellular and molecular investigation of the biology of CSMN and broader molecularly and developmentally related populations of SCPN in hSOD1G93A mice. Elucidating this CSMN/SCPN biology in ALS and for critical analysis of secondary fluorescent antibodies at 4°C overnight. Nuclear counterstain secondary fluorescent antibodies were used: FG (1:500, Millipore Bioscience Research Reagents), CD68 (1:100, Millipore Bioscience Research Reagents), cleaved caspase 3 (1:250, Cell Signaling), parvalbumin (PV; 1:500, Cruz Biotechnology), and perfused with cold 0.1 M PBS supplemented with heparin, followed by cold 4% paraformaldehyde (PFA) in 0.1 M PBS. To further investigate whether degeneration of other neocortical projection neurons with equivalently long axons [most notably, interhemispheric callosal projection neurons (CPN)] occurs, in a subset of experiments dual CPN and CSMN retrograde labeling was performed in hSOD1G93A and WT mice at P30, and mice were perfused at P120. CPN were retrogradely labeled via stereotactic injection of green fluorescent microspheres into contralateral cortex (250 nl/mouse), and CSMN were retrogradely labeled via stereotactic injection of red fluorescent microspheres (250 nl/mouse) into the cervical region (C4–C6) of the corticospinal tract within the dorsal funiculus of the spinal cord. Brains were postfixed in 4% PFA overnight, and 40 μm thick coronal sections were cut on a Leica VT 1000 S vibratome.

**Materials and Methods**

**Mice.** Male and female wild-type (WT), hemizygous transgenic mice overexpressing the wild-type human SOD1 gene (hSOD1WT), and hemizygous transgenic mice expressing the SOD1 gene containing the G93A mutation in hSOD1G93A mice (hSOD1G93A or hSOD1WT, respectively) were used. Thy1-YFP;WT mice were used as controls. All mouse studies were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee and performed in accordance with institutional, federal, and National Institutes of Health guidelines.

**CSMN labeling and tissue collection.** CSMN were retrogradely labeled via stereotactic FluoroGold injections (2% FG, 250 nl/mouse) into the cervical region (C4–C6) of the corticospinal tract within the dorsal funiculus of the spinal cord at distinct, phenotypically distinguishable times defined previously (Gurney et al., 1994; Tu et al., 1996; Hall et al., 1998; Cleveland and Rothstein, 2001; Wengenack et al., 2004; Hegedus et al., 2007) as P20, “early”; P50, “symptomatic”; and P110, “end stage.” In a subset of experiments, mice injected at P50 were perfused at P120 to distinguish between genuine CSMN degeneration and potential appearance of reduced FG labeling due to defects in axonal transport (Fig. 1A, “a”). Ten days after FluoroGold injection, mice were deeply anesthetized and perfused with cold 0.1 M PBS supplemented with heparin, followed by cold 4% paraformaldehyde (PFA) in 0.1 M PBS. To further investigate whether degeneration of other neocortical projection neurons with equivalently long axons [most notably, interhemispheric callosal projection neurons (CPN)] occurs, in a subset of experiments dual CPN and CSMN retrograde labeling was performed in hSOD1G93A and WT mice at P30, and mice were perfused at P120. CPN were retrogradely labeled via stereotactic injection of green fluorescent microspheres into contralateral cortex (250 nl/mouse), and CSMN were retrogradely labeled via stereotactic injection of red fluorescent microspheres (250 nl/mouse) into the cervical region (C4–C6) of the corticospinal tract within the dorsal funiculus of the spinal cord. Brains were postfixed in 4% PFA overnight, and 40 μm thick coronal sections were cut on a Leica VT 1000 S vibratome.

**Immunocytochemistry, Nissl staining, and in situ hybridization.** All immunocytochemical procedures were performed on every twelfth tissue section of 40-μm-thick coronal sections. The following primary antibodies were used: FG (1:500, Millipore Bioscience Research Reagents), CTIP2 (1:200, Calbiochem), LMO4 (1:200, Santa Cruz Biotechnology), cleaved caspase 3 (1:250, Cell Signalling), parvalbumin (PV; 1:500, Sigma), neuropeptide Y (NPY; 1:500, Immunostar), GFAP (1:200, Santa Cruz Biotechnology), CD68 (1:100, Millipore Bioscience Research Reagents), and FoxP2 (1:250, Abcam). Appropriate secondary fluorescent antibodies (1:500, Alexa 488 and 546, Invitrogen) were applied in blocking solution at room temperature, in the dark, for 2–4 h. CTIP2 and LMO4 immunocytochemical analysis was enhanced by increased primary antibody incubation (2 d) at 4°C, and 1:200 dilution was used for secondary fluorescent antibodies at 4°C overnight. Nuclear counterstain (ToPro; 1:5000 in 0.1 M PBS) was used to investigate nuclear pyknosis. Hybridization was performed using the following: 1 μg probe was added to a denatured in situ hybridization cocktail (Roche) and was hybridized for 24 hr at 50°C, containing 4% dextran sulfate, 0.1× standard saline citrate, 0.5% sodium dodecyl sulfate, 2 × 106 molecules/ml of riboflavin isomer 4′-dUTP, and 40 μm thick, coronal) isolated from WT, hSOD1WT, and hSOD1G93A mice. Cry-mur, IgGp1, Cax2, Fec2, and PlexinD1 probes were prepared, and in situ hybridization analysis was performed as previously described (Molyneaux et al., 2005, 2009).

**Imaging, quantification, and statistical analysis.** Sections were mounted on slides and analyzed using a Nikon E1000 fluorescence microscope equipped with an X-Cite 120 illuminator (EXFO), and images were acquired using a cooled charge-coupled device digital camera (Q Imaging Retiga EX). Confocal images were collected using a Bio-Rad Radiance 2100 Rainbow laser scanning confocal microscope based on a Nikon E800 microscope. Quantitative analyses were performed on three matched rostrocaudal sections separated by ~480 μm each [every ~12 sections; 40 μm section thickness (supplemental Fig. 1, available at www.jneurosci.org as supplemental material)]. Three adjacent fields defined by the 20× objective encompass the mediolateral extent of retrogradely labeled CSMN; the same systematically located fields were used for analysis in all mice. CSMN were counted only if their soma and apical dendrite were both visualized in the same thick 40 μm section. Representative sections and the locations of the three systematically located fields used for quantification of CSMN number and soma diameter in WT (P30, n = 5; P60, n = 5; P120, n = 5), hSOD1WT (P30, n = 5; P60, n = 5; P120, n = 5), and hSOD1G93A or hSOD1WT transgenic mice, respectively. Thy1-YFP;WT mice were used as controls. All mouse studies were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee and performed in accordance with institutional, federal, and National Institutes of Health guidelines.
hSOD1 G93A (P30, n = 5; P60, n = 5; P120, n = 8) mice are shown in supplemental Figure 1 (available at www.jneurosci.org as supplemental material). For each mouse, a total of nine fields were counted (three fields in each of three sections), and numbers were averaged. All statistical analyses were performed with INSTAT software (version 3.0a; Graphpad); parametric and nonparametric multiple-comparison tests, such as Bonferroni and Tukey tests, were used where appropriate, with a minimum significance level set at \( p < 0.05 \).

Quantification of interneuron number was performed based on expression of PV and NPY. Three comparable 40 \( \mu \)m coronal sections that span the motor cortex (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) from WT (P120, n = 2) and hSOD1 G93A (P120, n = 3) mice were analyzed. To perform unbiased and inclusive quantitative analysis, the cortex was divided into three equal sectors, mediolaterally/radially defined by equal 30° arcs. All neurons with both full cell bodies and immunopositivity for PV and NPY were counted in
each sector. All counts were performed “blinded” to the genotype of the sample by two independent observers. Interobserver variability was extremely low, with <3% variation between independent measurements.

To investigate the corticospinal tracts of Thy1-YFP:hSOD1 G93A mice (P30, n = 2; P60, n = 3; P120, n = 4) and Thy1-YFP:hSOD1 WT mice (P30, n = 3; P60, n = 3; P120, n = 3), spinal cords were isolated intact, and the cervical and lumbar segments of the spinal cords were sectioned axially (50 μm section thickness), whereas the thoracic segment was sectioned sagittally (150 μm section thickness). All thoracic segments containing axons were mounted on slides, and images of all sections that contained corticospinal tract axons were digitally collapsed to obtain a two-dimensional image to indicate the whole extent of the corticospinal tract. Representative axial sections of cervical and lumbar spinal cord were also imaged.

Results

CSMN progressively degenerate in hSOD1 G93A mice

We used a combination of anatomical and molecular approaches to identify, quantify, and assess potential vulnerability and progressive degeneration of CSMN. We retrogradely labeled CSMN by FG injection into the corticospinal tract within the dorsal funiculus of the C4–C6 region of the spinal cord (Fig. 1A). CSMN were retrogradely labeled at P20 for analysis at P30 (presymptomatic), at P50 for analysis at P60 (symptomatic), and at P100 for analysis at P120 (end-stage). In a subset of experiments designed to distinguish between genuine CSMN degeneration and potential appearance of reduced FG labeling due to defects in axonal transport, CSMN were retrogradely labeled at P50 and analyzed at P120 (Fig. 1A, “#”). P30, P60, and P120 times were chosen based on published and well-established stages of disease progression with respect to both observed behavioral phenotypic changes and spinal motor neuron degeneration in hSOD1 G93A mice (Gurney et al., 1994; Hegedus et al., 2007). CSMN identity was established unequivocally by retrograde labeling from axon projections in the spinal cord, and verified based on (1) neuronal location in the primary motor cortex, (2) distinctive large CSMN morphology, and (3) expression of multiple recently identified positive and negative molecular markers that are either specifically expressed or excluded by CSMN (and related SCPN in some cases). Retrogradely labeled CSMN were confirmed to reside in layer V of the motor cortex and displayed distinct somatodendritic morphology of CSMN: they have a large pyramidal cell body and a long apical dendrite (Fig. 1B). Retrogradely labeled neurons expressed CTIP2 (Arlotta et al., 2005), a CSMN-specific (and cortico-brainstem; together, subcerebral-specific) transcription factor in layer V (n = 150, 100%) (Fig. 1C), and did not express LMO4, a transcription factor excluded from CSMN in layer V (n = 120, 0%) (Fig. 1D), confirming their CSMN identity. Although both CTIP2 and LMO4 expression levels are highest during development, and reduce with age, they remain specific and detectable (Fig. 1C,D) (see Materials and Methods). Homogeneity in the background of the WT, hSOD1 WT, and hSOD1 G93A mice, and the consistent copy number of transgenic gene expression (30 ± 2) in hSOD1 WT and hSOD1 G93A mice resulted in highly reproducible timing of disease onset and progression, enabling detailed and reproducible qualitative and quantitative analysis of CSMN degeneration at specific times during disease progression.

Qualitative analysis of retrogradely labeled CSMN in layer V of motor cortex in P30, P60, and P120 WT, hSOD1 WT, and hSOD1 G93A mice reveals dramatic CSMN vulnerability and marked degeneration in P120 hSOD1 G93A mice compared with WT and hSOD1 WT controls, present but more subtle at P30 and P60 (Fig. 1E–H). Quantitative analysis of CSMN soma diameter and neuron number at P30, P60, and P120 indicates progressive degeneration of CSMN only in hSOD1 G93A mice (Fig. 1G,H; supplemental Fig. 1, available at www.jneurosci.org as supplemental material). CSMN soma diameter in hSOD1 G93A mice significantly decreases, even during the “presymptomatic” stage (P30), and this decrease becomes even more striking with disease progression (Fig. 1G) (14 ± 2 μm at P30; 10 ± 2 μm at P60; 7 ± 2 μm at P120). In contrast, neither WT CSMN (19 ± 2 μm at P30; 20 ± 2 μm at P60; 20 ± 2 μm at P120) nor hSOD1 WT CSMN (18 ± 2 μm at P30; 19 ± 3 μm at P60; 21 ± 2 μm at P120) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) show any reduction in soma diameter.

Quantitative analysis of the number of CSMN in motor cortex indicates a progressive and dramatic degeneration of CSMN in hSOD1 G93A mice beginning by P30 (Fig. 1H) (13 ± 1 per 20× field at P30, n = 5 mice; 10 ± 1 at P60, n = 6 mice; 5 ± 2 at P120, n = 8 mice; 6 ± 1 at P120, injected at P50, n = 3 mice) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). In contrast, CSMN of neither WT (15 ± 1 at P30, n = 5 mice; 14 ± 2 ± 1 at P60, n = 5 mice; 16 ± 2 at P120, n = 6 mice) nor hSOD1 WT mice (16 ± 1 at P30, n = 4 mice; 15 ± 2 at P60, n = 5 mice; 14 ± 1 at P120, n = 5 mice) demonstrate neuronal degeneration. At P120, hSOD1 G93A mice show identical numbers of retrogradely labeled CSMN, regardless of the time of retrograde labeling (5 ± 2 at P120, injected at P100, n = 8 mice; 6 ± 1 at P120, injected at P50, n = 3 mice), strongly indicating that CSMN undergo genuine somatic degeneration, and that the reduced number of retrogradely labeled CSMN is not due to confounding variables related to retrograde transport. In contrast, rubospinal neurons, located in the red nucleus, and projecting to the cervical spinal cord (and thus also retrogradely labeled from the cervical spinal cord FG injections) are normal in number and morphology in hSOD1 G93A mice (as well as in hSOD1 WT and WT mice) at all ages including P120, reinforcing that degeneration is specific to CSMN in motor cortex in hSOD1 G93A mice (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

CSMN degeneration occurs via apoptosis

Investigation of CSMN death at all three stages of disease progression, using Nissl staining, analysis for nuclear pyknosis, and cleaved caspase-3 (CC-3) expression, reveals that CSMN death is apoptotic, and starts by P30 in hSOD1 G93A mice (Fig. 2). Nuclear pyknosis is an early sign of apoptotic cell death (de Rivero Vaccari et al., 2006), and CC-3 expression is detected in cells undergoing active apoptosis, although the time it takes CC-3-positive neurons to ultimately die is reported to take substantially longer in vivo than in vitro (Yang et al., 1998; Arai et al., 2005). Apoptotic degeneration in hSOD1 G93A spinal motor neurons has been documented previously (Li et al., 2000; Przedborski, 2004; Wengenack et al., 2004), but CSMN were not investigated in detail. Here, all three analyses (Nissl, nuclear pyknosis, CC-3) reveal a wave of apoptotic CSMN death that starts by P30 in hSOD1 G93A mice. In striking contrast, WT and hSOD1 WT CSMN do not demonstrate neuronal death by any of these three modes of analysis at any stage investigated. At P30, apoptosis is visible by nuclear pyknosis (Fig. 2A) (40 ± 5% of hSOD1 G93A CSMN, n = 290; 1 ± 1% of WT CSMN, n = 271; 3 ± 1% hSOD1 WT CSMN, n = 264) (Fig. 2A,D) and reduced soma size (Fig. 1G), and CC-3 expression is detected in some CSMN in hSOD1 G93A mice (2 ± 1%, n = 148), but not in WT or hSOD1 WT. To further investigate whether nuclear pyknosis, and thus neuronal degeneration, might begin even earlier than P30, we quantified nuclear pyknosis of CTIP2-
positive CSMN in layer V of motor cortex at P5, P10, and P15 compared with WT control CSMN at these same ages. These analyses reveal that 0% of WT CSMN display pyknotic nuclei at any of these early ages (P5, n = 105; P10, n = 101; P15, n = 110), whereas 2% of P5 hSOD1<sup>G93A</sup> CSMN display pyknosis, n = 114; increasing to 4 ± 1% at P10, n = 147; and to 7 ± 1% at P15, n = 112. At P60, the percentages of CSMN with pyknotic nuclei (64 ± 3%, n = 243) or expressing CC-3 (9 ± 2%, n = 140) are substantially increased (Fig. 2B, D, E). At P120, CSMN degeneration in hSOD1<sup>G93A</sup> mice is even more dramatic, with 7 ± 3% of CSMN expressing CC-3, 61 ± 3% with pyknotic nuclei, and near absence of large pyramidal neurons in layer V of motor cortex by Nissl staining (Fig. 2C). It is likely that CSMN detected at each age would include CSMN (1) already committed to die via apoptosis, (2) already dysfunctional and in the process of cell death, and (3) dead but not yet cleared from the tissue. These data suggest that many retrogradely labeled somata of CSMN detected in the cortex at P30, P60, and/or P120 are already less viable (likely already undergoing degeneration), but they are either not yet dead or not yet cleared from the tissue. Together, these results indicate by multiple independent criteria that progressive CSMN degeneration occurs in hSOD1<sup>G93A</sup> mice, beginning early during the classic...
Figure 3. Degeneration is specific to CSMN and SCPN. A–E. In situ hybridization analysis of Fezf2 (A), Cry-mu (B), PlexinD1 (C), Cux2 (D), and Igfbp4 (E) on coronal sections of hSOD1WT and hSOD1G93A cortex at P120. The boxed areas (i–iv) in the low-magnification images are enlarged to the right; roman numerals indicate the layers of motor cortex. Fezf2 is expressed at high levels in layer V of motor cortex (i) and nonmotor areas (iii) only in hSOD1WT mice. Fezf2 expression is dramatically reduced in the motor cortex (CSMN) (ii) and in the nonmotor areas of the cortex (SCPN) (Figure legend continues.)
Broader degeneration of developmentally related nonmotor subcerebral projection neurons occurs in hSOD1<sup>G93A</sup> mice

To further investigate whether neocortical neuronal degeneration is specific to CSMN, or might involve other populations of cortical projection neurons and/or interneurons, we investigated the neuronal subtype identity and specificity of degeneration, using laminar and neuron type-specific molecular markers. We used a combination of immunocytochemical and in situ hybridization analyses in hSOD1<sup>G93A</sup> and hSOD1<sup>WT</sup> mice at P120. The results indicate that neuronal degeneration extends specifically to the developmentally related populations of motor and nonmotor, associative subcerebral projection neurons (SCPN), which include CSMN in the motor cortex. Neurons in other layers of the neocortex, and other neuron populations in layer V, are not affected.

Each molecular marker analyzed, both for CSMN/SCPN and for alternate neuron subtypes and lamina, confirms the specificity of CSMN/SCPN degeneration in the motor cortex of hSOD1<sup>G93A</sup> mice. Fezf2, a transcription factor required for CSMN/SCPN specification and development, and expressed at high levels in CSMN/SCPN throughout life (Molyneaux et al., 2005; Molyneaux et al., 2007), has normal expression in hSOD1<sup>WT</sup> mice at P120 (Fig. 3A). In contrast, Fezf2 expression in motor cortex of hSOD1<sup>G93A</sup> mice indicates dramatic loss of CSMN at P120 (Fig. 3Aii,iii). Interestingly, analysis of Fezf2 expression in nonmotor areas of neocortex indicate additional involvement, but less striking loss, of the broader population of SCPN (Fig. 3Aiii,iv), which contribute to nonmotor cognitive, association, and integrative sensory systems in the cortex. Cry-mu, which is expressed at high levels by CSMN during development, and at very low levels by a subset of corticofugal neurons located in layer V (Molyneaux et al., 2005; Molyneaux et al., 2007), displays normal expression in hSOD1<sup>WT</sup> mice at P120 (Fig. 3Bii). In striking contrast, cry-mu expression confirms dramatic loss of CSMN in motor cortex in hSOD1<sup>G93A</sup> mice (Fig. 3Bii). With regard to non-CSMN/SCPN populations, PlexinD1 (Fig. 3C) and Cux2 (Fig. 3D), both expressed in neuron populations in superficial layers of the neocortex during development through adulthood (Molyneaux et al., 2007), do not indicate any difference in superficial layer neuron populations in hSOD1<sup>G93A</sup> versus hSOD1<sup>WT</sup> mice. To further differentiate true CSMN/SCPN neuronal loss from simple gene dysregulation, Igfbp4, expressed at high levels by CSMN during development, and at lower levels in layer II/III and layer VI neurons in the cortex (Molyneaux et al., 2007), displays normal expression in all populations in hSOD1<sup>WT</sup> mice at P120 (Fig. 3E). In contrast, in hSOD1<sup>G93A</sup> mice, Igfbp4 expression is sharply reduced in layer V (confirming CSMN/SCPN loss), while its expression is not affected in other neuron populations located in neocortical layers II/III and VI (Fig. 3E), further reinforcing the specificity of CSMN/SCPN degeneration in layer V of motor cortex. To further consider whether neuronal degeneration is specific to CSMN/SCPN among the broader corticofugal populations (including, e.g., corticothalamic neurons), analysis of deepest layer VI of cortex for FoxP2 expression does not indicate any difference between hSOD1<sup>G93A</sup> and hSOD1<sup>WT</sup> mice (Fig. 3F). Similarly, LMO4, a transcription factor expressed by callosal projection neurons (inter-hemispheric, non-CSMN), interneurons, and other populations in layer V, but excluded from CSMN in layer V of the cortex (Arlotta et al., 2005; Molyneaux et al., 2005), is unchanged between hSOD1<sup>G93A</sup> and hSOD1<sup>WT</sup> mice (Fig. 3Gi,ii). In addition, dual retrograde labeling of CPN and CSMN at P50 in hSOD1<sup>G93A</sup> and WT mice with perfusion at P120 indicates 1) no loss of CPN in hSOD1<sup>G93A</sup> mice, 2) striking CSMN/SCPN-specific loss in hSOD1<sup>G93A</sup> mice (again, labeled early to exclude retrograde transport as a confounding variable), 3) specificity of CSMN degeneration in motor cortex of hSOD1<sup>G93A</sup> mice (supplemental Fig. 3, available at www.jneurosci.org as supplemental material).

Interneurons are not similarly affected in hSOD1<sup>G93A</sup> mice

To investigate whether vulnerability and degeneration is restricted to CSMN/SCPN in the cortex of hSOD1<sup>G93A</sup> mice, or whether nonprojection neuron populations (interneurons) are affected, we examined a broad variety of interneurons based on the interneuron molecular markers parvalbumin and neuropeptide Y (NPY) in both hSOD1<sup>G93A</sup> (n = 3) and WT (n = 2) mice during the end-stage of disease progression. The results indicate that cortical interneuron populations are not apparently affected in hSOD1<sup>G93A</sup> mice (Fig. 4). The number of parvalbumin-expressing interneurons does not differ substantially or significantly between hSOD1<sup>G93A</sup> and WT mice, in any of the three mediolateral regions (Fig. 4A–C). Further, cellular morphologies of parvalbumin-positive cortical interneurons appear unchanged between WT and hSOD1<sup>G93A</sup> mice, without suggestion of neuronal degeneration (Fig. 4D, E). Similar results were obtained for NPY-expressing interneurons (data not shown). A prior report suggested the possibility of interneuron degeneration in the cortex based on reduced cortical inhibition in ALS patients (Zieman et al., 1997), but our analysis in hSOD1<sup>G93A</sup> mice does not identify interneuron loss in these model mice. One recent study suggested increased cortical interneuron numbers in ALS mice, based on immunocytochemical analysis and applications of modeling (Minciacchi et al., 2009), but our results using broad mediolateral and rostrocaudal analysis do not identify such an increase. While it still remains possible that slight differences between the transgenic mice exist in localized areas, potentially secondary to degeneration of interneurons’ CSMN/SCPN synaptic partners, we find no significant difference in interneurons more broadly between WT and hSOD1<sup>G93A</sup> mouse neocortex.

Astrogliosis and microgliosis are present late during CSMN/SCPN degeneration

Activated astrocytes have been implicated in disease pathogenesis in ALS and have been detected both in ALS patient brains and spinal cords, and in spinal cords of hSOD1<sup>G93A</sup> mice. To investigate whether astrogliosis (and, potentially, microglial activation) occurs in hSOD1<sup>G93A</sup> mouse neocortex, and whether astrogliosis...
Astrogliosis and microgliosis occur specifically in CD68 immunocytochemical analysis, respectively. We tested the presence of activated astrocytes and/or microglia in hSOD1G93A (P30, n = 3; P60, n = 3; P120, n = 4) and in hSOD1WT (P30, n = 2; P60, n = 2; P120, n = 3) mice using GFAP and CD68 immunocytochemical analysis, respectively. We find that astrogliosis and microgliosis occur specifically in hSOD1G93A mice (Fig. 5A–I, M) and not in hSOD1WT mice (Fig. 5J–L). It occurs mainly at late stages of disease progression in hSOD1G93A mice (Fig. 5G–I) and is not restricted to motor cortex (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). These results suggest that CSMN/SCMN vulnerability and degeneration is initiated before astrogliosis and microgliosis in the neocortex of hSOD1G93A mice.

The corticospinal tract degenerates in hSOD1G93A mice

To investigate the time course of corticospinal tract (CST) degeneration in hSOD1G93A mice that is the predicted accompaniment of the CSMN degeneration we identified in these mice, we applied genetic CST labeling using hSOD1 G93A, hSOD1 WT, and Thy1-YFP;hSOD1G93A mice by serial breeding (see Materials and Methods) (Fig. 6B). Results from Thy1-YFP;hSOD1G93A (P30, n = 2; P60, n = 3; P120, n = 3) (Fig. 6A, B) and Thy1-YFP;hSOD1WT mice (P30, n = 2; P60, n = 4; P120, n = 5) (Fig. 6B) indicate that corticospinal tract degeneration is specific to Thy1-YFP;hSOD1G93A mice. Degeneration is most dramatic between P60 and P120 (Fig. 6D–F). CST innervation of the lumbar spinal cord is essentially absent in Thy1-YFP;hSOD1G93A mice by the late stages of disease progression (Fig. 6Eii), and there is substantial loss of the cervical corticospinal tract (Fig. 6Ei). These results indicate that CSMN somal degeneration that occurs quite early is followed by profound corticospinal tract degeneration by late stages in hSOD1G93A mice.

Discussion

Together, using a combination of anatomic, cellular, transgenic labeling, and newly available neuronal subtype-specific molecular analyses of both laminar and neuronal subtype identity, these results demonstrate that early and neuron type-specific CSMN/SCPN (both motor and nonmotor) neuronal degeneration is faithfully recapitulated in hSOD1G93A transgenic ALS mice. Other neuron populations in other layers of the neocortex, other neuron populations within layer V, and interpersed cortical interneurons do not undergo such degeneration. Thus, hSOD1G93A mice develop specific CSMN/SCPN degeneration that mirrors the cortical pathology found in human ALS, and nonmotor SCPN degeneration might explain some or much of the nonmotor cortical pathology identified in ALS. This neuron type-specific degeneration in hSOD1G93A mice begins exceptionally early, in the presymptomatic stage, coincident with the earliest degeneration of spinal motor neurons (Gurney et al., 1994; Tu et al., 1996; Hall et al., 1998; Cleveland and Rothstein, 2001; Bruijn et al., 2004; Wengenack et al., 2004; Hegedus et al., 2007). These results are consistent with longstanding clinical evidence and more recent human magnetic resonance imaging...
studies that clearly indicate that CSMN in motor cortex are affected at early stages of ALS (Brown, 2005; Graham et al., 2004; Stewart et al., 2006). The data reveal early events of CSMN degeneration by P30, with progressive CSMN degeneration occurring via apoptosis. Neocortical neuron degeneration is specific to CSMN/SCPN in the cerebral cortex of hSOD1G93A mice. In contrast, other cortical neuron populations do not undergo such degeneration—other cortical projection neuron populations in other layers of the cortex, other projection neuron populations within cortical layer V, or interneurons. CSMN undergo true cellular degeneration, not simply defects in retrograde axonal transport, shown to be separable issues by multistage retrograde labeling experiments. CSMN-specific degeneration is accompanied by striking corticospinal tract degeneration later in disease progression, revealed by transgenic labeling of corticospinal tract axons in Thy1-YFP;hSOD1G93A mice. These experiments and findings provide a foundation for detailed investigation of CSMN/SCPN degeneration in hSOD1G93A mice.

Figure 5. Astrogliosis and microgliosis is observed in hSOD1G93A mice. A–I, Astrogliosis in motor cortex of hSOD1G93A mice at P30 (A–C), P60 (D, E), and P120 (G–I). The boxed areas in the low-magnification images are enlarged to the right. Although progressive CSMN degeneration is observed in hSOD1G93A mice at P30 to P60 (B, C, E, F) and P120 (B, C, E, F, H, I), activated astrocytes were first detected by GFAP expression later, here shown at P120 (H, I). Astrogliosis was specific to hSOD1G93A mice, and hSOD1WT mice do not display activated astrocytes at P120 (J–L). Similarly, activated microglia were detected by CD68 expression only in hSOD1G93A cortex (M) and not in hSOD1WT cortex (N) at P120.
It is quite notable that CSMN degeneration is accompanied by degeneration of the related broader category of SCPN. Types of SCPN other than CSMN are molecularly and developmentally closely related to CSMN, and their coincident degeneration might be in part responsible for the broader cortical pathology in cognitive, association, and even sensory systems in many ALS cases. This further links the findings in hSOD1\textsuperscript{G93A} mice to this cognition, association, and even sensory systems in many ALS patients. Such investigations will likely also elucidate mechanisms of CSMN/SCPN degeneration in other upper motor neuron neurodegenerative diseases, such as hereditary spastic paraplegia and primary lateral sclerosis.

CSMN and SMN degenerate independently, but their degeneration is potentially mechanistically related. CSMN and SMN are located at distant locations in the CNS, have very different developmental histories, and are positioned in distinct but potentially related microenvironments (with related glutamatergic synapses, transmission, and functions of surrounding astroglia, and potentially some shared channel properties). However, their progressive and specific degeneration indicates the relevance and importance of potentially common biology between these upper and lower motor neurons in ALS.

Sufficient information is developing to begin to elucidate common and/or unique biology between CSMN and SMN during development, and in ALS and related neurodegenerative diseases. There has been deep and important work on the differentiation, development, and maturation of spinal motor neurons (Tanabe and Jessell, 1996; Ericson et al., 1997; Arber et al., 1999; Briscoe et al., 1999; Pierani et al., 2001; Wichterle et al., 2002; William et al., 2003; Dasen et al., 2003, 2005; Kania and Jessell, 2003; Lanuza et al., 2004; Fox et al., 2007; Azim et al., 2008; Azim et al., 2009a,b; Tomassy et al., 2010). In addition, cellular and molecular controls over differentiation, development, and maturation of CSMN/SCPN and other populations of neocortical projection neurons are beginning to emerge (Arlotta et al., 2005; B. Chen et al., 2005; J. G. Chen et al., 2005; Tomassy et al., 2010; Ozdinler and Macklis, 2006; Joshi et al., 2008; Lai et al., 2008; Azim et al., 2009a,b; Tomassy et al., 2010). Moreover,
there have been multiple recent studies investigating early defects in corticospinal motor neuron circuitry in the cerebral cortex of ALS patients and the progressive cortical pathology with respect to spinal motor neuron degeneration (Vucic et al., 2008; Sivak et al., 2010; van der Graaff et al., 2010; Mohammadi et al., 2011). Emerging evidence also indicates the relevance of SOD1 pathology in at least some sporadic ALS cases and extends the importance of understanding SOD1-mediated pathology well beyond familial ALS (Bosco et al., 2010). These studies, together with increasing understanding regarding the biology of both upper and lower motor neurons, might contribute toward development of future diagnostic and therapeutic approaches.

Understanding cellular and molecular mechanisms of cell type-specific degeneration in neurodegenerative diseases is of major and important interest toward both prevention and therapy. Together, our investigations establish and clarify the central, early, specific, and independent involvement of CSMN/SCPN in hSOD1G93A mice, and develop neuron type-specific approaches for analysis of the upper motor neuron component of neurodegeneration and its potential prevention or therapy in hSOD1G93A mice and other rodent models of ALS.

References

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