Published in final edited form as:

Neurobiol Dis. 2012 August ; 47(2): 174–183. doi:10.1016/j.nbd.2012.03.036.

AAV2 mediated retrograde transduction of corticospinal motor neurons reveals initial and selective apical dendrite degeneration in ALS

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Abstract

Corticospinal motor neurons (CSMN) are the cortical component of motor neuron circuitry, which controls voluntary movement and degenerates in diseases such as amyotrophic lateral sclerosis, primary lateral sclerosis and hereditary spastic paraplegia. By using dual labeling combined with molecular marker analysis, we identified AAV2-2 mediated retrograde transduction as an effective approach to selectively target CSMN without affecting other neuron populations both in wild-type and hSOD1^{G93A} transgenic ALS mice. This approach reveals very precise details of cytoarchitectural defects within vulnerable neurons *in vivo*. We report that CSMN vulnerability is marked by selective degeneration of apical dendrites especially in layer II/III of the hSOD1^{G93A} mouse motor cortex, where cortical input to CSMN function is vastly modulated. While our findings confirm the presence of astrogliosis and microglia activation, they do not lend support to their direct role for the initiation of CSMN vulnerability. This study enables development of targeted gene replacement strategies to CSMN in the cerebral cortex, and reveals CSMN cortical modulation defects as a potential cause of neuronal vulnerability in ALS.

Keywords

Corticospinal motor neurons; ALS; Gene therapy

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Available online on ScienceDirect (www.sciencedirect.com).

Supplementary data associated with this article can be found, in the online version, at dx.doi.org/10.1016/j.nbd.2012.03.036.

Introduction

In amyotrophic lateral sclerosis (ALS), unlike other motor neuron diseases, both the cortical and spinal components of motor neuron circuitry progressively degenerate (Brown and Robberecht, 2001; Ravits et al., 2007). Therefore, bringing effective treatments to ALS requires targeting both the spinal motor neurons (SMN) in the spinal cord and the corticospinal motor neurons (CSMN) in the motor cortex. CSMN degeneration results in lack of cortical input and impairs voluntary movement in other motor neuron disorders, such as primary lateral sclerosis and hereditary spastic paraplegia (Fink, 2002, 2006; Rainier et al., 2003). Therefore, due to the central involvement of CSMN in motor neuron circuitry and their selective vulnerability and degeneration in various diseases, it is imperative to understand cellular mechanisms for CSMN vulnerability and to develop CSMN directed gene delivery approaches in the cerebral cortex.

Viral vectors offer a great tool for gene therapy and replacement studies in CNS (Kootstra and Verma, 2003; Verma and Weitzman, 2005). Recombinant, adeno-associated viruses (AAV) are ideal candidates for retrograde transduction of projection neurons and are extensively used to target SMN. AAV2-IGF-1 injected into the muscle of hSOD1^{G93A} ALS transgenic mice, one of the best-characterized mouse models of ALS (Gurney et al., 1994), retrogradely transduced SMN and increased life span (Kaspar et al., 2003). Intravascular administration of AAV9 (Foust et al., 2009) and intrathecal injections of AAV6 and AAV9 at the level of the lumbar spinal cord transduced SMN throughout the spinal cord (Snyder et al., 2011). Intramuscular delivery of AAV6 encoding silencer shSOD1 RNA transduced SMN, but failed to alter disease course in the hSOD1^{G93A} mouse (Towne et al., 2011). Moreover, CST fibers were labeled and mixed neuron populations were transduced by direct AAV injection into the motor cortex (Hutson et al., 2011), but effective retrograde transduction of CSMN has never been achieved, even though AAV-mediated gene therapy approaches have been considered for ALS (Nizzardo et al., 2011).

In an effort to build gene delivery approaches selectively targeting CSMN and to reveal cytoarchitectural changes that correlate with cellular mechanisms underlying CSMN vulnerability, we used an AAV-mediated retrograde transduction strategy coupled with cellular analysis. We investigated whether a distinct set of AAV serotypes can be used to retrogradely transduce CSMN following precise injection into the corticospinal tract (CST), without affecting other spinal cord neurons and circuitries. AAV2-2 was found to be most effective in CSMN gene delivery in both wild-type (WT) and hSOD1^{G93A} transgenic ALS mice, which show early and selective CSMN degeneration (Ozdinler et al., 2011). Retrograde transduction of CSMN with an enhanced green fluorescent protein (eGFP) gene fully revealed the cytoarchitecture of CSMN at a level of detail not previously possible by other retrograde labeling approaches (Hains et al., 2003; Ozdinler et al., 2011; Shokouhi et al., 2010).

We report spine loss, as well as severe and selective apical dendrite degeneration, especially in layer II/III of motor cortex in the hSOD1^{G93A} transgenic ALS mice at P60, whereas basal dendrites are unaffected. Cellular interaction studies of CSMN with astrocytes and microglia, cell types that are extensively studied with respect to disease initiation and

progression (Alexianu et al., 2001; Hall et al., 1998; Levine et al., 1999), revealed close cell–cell interactions especially with degenerating hSOD1^{G93A} CSMN both at the soma and apical dendrite. Our results set the stage for future targeted gene delivery approaches to CSMN and validate the use of hSOD1^{G93A} mouse for further gene therapy approaches for ALS. In addition, our findings suggest that an intrinsic cellular defect affecting distal apical dendrites, especially in layer II/III of motor cortex, underlies connectivity and modulation deficiencies of CSMN as a potential mechanism for their vulnerability and degeneration.

Materials and methods

Mice

All procedures were approved by the Northwestern University Animal Care and Use Committee and conformed to the standards of the National Institutes of Health. WT and hSOD1^{G93A} transgenic ALS mice, in C57BL/6 background, were obtained from Jackson laboratories (Gurney et al., 1994). Genotypes of mice were determined as described by the vendor.

Adeno-associated virus (AAV) serotype generation

AAV vectors with different serotypes were generated by the University of Pennsylvania Vector Core facility by triple transfection of subcon-fluent HEK293 cells using three plasmids: an AAV trans-plasmid encoding one of either AAV1, 2, 5, 6, 7, 8 or 9 capsid, an adenovirus helper plasmid, pDF6, and an AAV cis shuttle plasmid expressing eGFP driven by a CMV promoter (pENN.AAV.CMV.PI.eGFP.WPRE.bGH). The culture medium containing the vector particles was collected, concentrated by tangential flow filtration and purified by iodixanol gradient ultracentrifugation followed by further concentration and buffer exchange as previously described (Lock et al., 2010).

CSMN retrograde transduction surgeries

Surgeries involving retrograde transduction of CSMN were performed on postnatal day 30 (P30) WT and hSOD1^{G93A} mice that were deeply anesthetized with isoflurane and placed onto a stereotaxic platform equipped with a nanojector (Drummond Scientific, PA). A small laminectomy at the cervical spinal cord (C2–C3) level was performed, and AAV serotypes and red fluorescent microspheres (Lumafluor Inc., FL) were mixed together and injected into the corticospinal tract (CST) that lies within the dorsal funiculus (df) at 0.3 mm depth. A volume of 621 nl containing 1.16×10^9 vector particles (v.p.) mixed with red fluorescent microspheres (Fig. 1A) was injected. Brain and spinal cord tissues were collected from WT and hSOD1^{G93A} mice at P60.

Tissue isolation and processing

Mice were deeply anesthetized by Ketamine (90 mg/kg) with Xylazine (10 mg/kg) and transcardially perfused with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). The brain and spinal cord were removed and were post-fixed (4% PFA, overnight) and kept in PBS-sodium azide (0.01%) at 4 °C. Coronal (50 µm) sections were collected in 12-well plates using a vibratome (Leica SV, 100T).

Immunocytochemical analysis

Anti-GFP (1:1000; Abcam), anti-CTIP2 (1:500; Abcam), anti-GFAP (1:2000; Invitrogen), anti-Iba1 (1:500; Wako), anti-CD11b (1:1000; Serotec) were used for immunocytochemical analysis as previously described (Jara et al., 2007). Appropriate fluorescent secondary antibodies (Molecular Probes) or biotinylated secondary antibodies in combination with avidin-biotin complex (ABC) solution (Vector Laboratories) were applied. DAB with ImmPACT DAB peroxidase substrate kit was used (Vector Laboratories).

Imaging

Sections were analyzed using a Nikon Eclipse TE2000-E fluorescence microscope equipped with Intensilight C-HGFI (Nikon). Epi-fluorescent images were acquired using Digital Sight DS-Qi1MC CCD camera (Nikon). Confocal images were collected using a Zeiss 510 Meta confocal microscope (Zeiss).

Quantification and statistical analysis

AAV mediated retrograde transduction efficiency of CSMN was determined by quantifying the percentage of eGFP-expressing (eGFP+) CSMN in the total population of CSMN exposed to the viral mixture (determined by the number of CSMN with red fluorescent micro-spheres). For each genotype three mice were used for each analysis. Three well-defined sections spanning the motor cortex (Bregma 0.86 mm, interaural 4.66 mm; Bregma 0.02 mm, interaural 3.82 mm; Bregma -1.22 mm, interaural 2.58 mm) of each mouse were used (Paxinos and Franklin, 2001). CSMN interaction with glial cells was investigated using cellular markers for astrocytes or microglia. Microglia and astrocytes that are in close proximity to CSMN soma and dendrites were quantified and analyzed based on their GFAP and Iba-1 expression and cell morphology (Supplementary Figs. 2A–B). Statistical differences were determined by one-way ANOVA followed by Tukey–Kramer's multiple comparison post-hoc parametric analysis. Soma area and number of primary basal dendrites were quantified using NIKON NIS Elements software. For spine density measurements, a 50 μ m segment of apical dendrites (in layer II/III, in the primary apical dendrite, Figs. 4D, H) and basal dendrites (in layer V, separated by 30 μ m from the soma) were selected and the total numbers of spines were counted. 6 WT and 5 hSOD1^{G93A} mice were used for quantitative analysis. Statistical differences were determined by two-tailed Student *t*-test. Statistically significant differences were taken at *p* 0.05 and values expressed as the mean $\pm$ SD (standard deviation).

Results

AAV-mediated retrograde transduction of CSMN

CSMN can be retrogradely labeled by red fluorescent microspheres or FluoroGold injections into CST at the C2–C3 level of the spinal cord in the postnatal mice (Ozdinler and Macklis, 2006; Ozdinler et al., 2011). Using this approach, we studied the possibility of targeted gene delivery to CSMN using AAV and determined the AAV serotype with the highest transduction efficiency in both WT and hSOD1^{G93A} mice. CST injections performed using matched-titers of AAV2–1, AAV2–2, AAV2–5, AAV2–6, AAV2–7, AAV2–8 or AAV2–9

together with red fluorescent microspheres demonstrate that the injection sites were restricted to the dorsal funiculus, the anatomical location of the CST in mouse (Figs. 1A–C). No transduction was detected in other cells/neurons surrounding the injection sites (Fig. 1C). CSMN were equally retrogradely labeled with fluorescent microspheres indicating comparable CST injections for all AAV serotypes tested, and eGFP+CSMN identity was confirmed by CTIP2 expression, a molecular marker expressed by CSMN in layer V of motor cortex (Arlotta et al., 2005). However, each AAV serotype displayed different retrograde transduction efficiency for CSMN 30 days post-injection (P60). AAV2–2 showed the highest level of transduction (Fig. 2A; $21.7 \pm 7\%$, $n=1582$ CSMN; $n=3$ mice). AAV2–5 and AAV2–9 (Figs. 2B,D) showed low levels of retrograde transduction (AAV2–5 transduced 1.5% of CSMN, $n=1008$ CSMN, $n=3$ mice; and AAV2–9 transduced 0.26% of CSMN, $n=1695$ CSMN, $n=3$ mice). In striking contrast, AAV2–8 (Fig. 2C) and AAV2–6 did not retrogradely transduce CSMN (Supplementary Fig. 1), and AAV2–1, and AAV2–7 displayed moderate to low levels of retrograde transduction (Supplementary Fig. 1). We next asked whether AAV2–2 is able to retrogradely transduce CSMN in the hSOD1^{G93A} mice where CSMN vulnerability and degeneration are studied (Ozdinler et al., 2011). AAV2–2 was injected into the CST of hSOD1^{G93A} mice at P30 to retrogradely transduce CSMN in the motor cortex. Effective and comparable retrograde transduction of hSOD1^{G93A} CSMN was observed at P60 and transduced CSMN were identified by the presence of red fluorescent microspheres and CTIP2 expression (Figs. 3A–C; $14.5 \pm 6\%$, $n=1498$ CSMN; $n=3$ mice); no statistical differences were detected compared with WT mice ($p>0.05$).

CSMN spine loss and apical dendrite degeneration in layer II/III

Enhancement of eGFP expression analysis by a DAB-based immunocytochemistry revealed details of CSMN neuroarchitecture. WT CSMN were characterized by dendritic branching and arborization especially in layer II/III of the motor cortex, with high spine density in those branches and in the apical dendrite (Figs. 4A,D). In striking contrast, hSOD1^{G93A} CSMN showed severe and selective degeneration of apical dendrites and a lack of spines, especially in layer II/III of motor cortex (Figs. 4E–H, M). Moreover, apical dendrites in hSOD1^{G93A} were observed to be vacuolated (56% in hSOD1^{G93A}, $n=288$ apical dendrites, $n=5$ mice; and 6% in WT, $n=521$ apical dendrites, $n=6$ mice) and some exhibited the morphology of retracted and dying back neurites, especially in layer IV (Fig. 4G, arrow). Analysis of primary apical dendrite spine density (Figs. 4D,H) in layer II/III revealed pronounced reduction in the hSOD1^{G93A} mice (hSOD1^{G93A}, 1.5 ± 0.1 spines/10 μm , $n=5$ mice; WT, 4.4 ± 0.1 spines/10 μm , $n=6$ mice; $p<0.05$, Fig. 4M). However, cellular degeneration was not detected in layer V (Figs. 4I,J). CSMN of both WT and hSOD1^{G93A} mice had comparable soma areas (hSOD1^{G93A} $177 \pm 7 \mu\text{m}^2$, $n=5$ mice; WT $185 \pm 6 \mu\text{m}^2$, $n=6$ mice Fig. 4K) and average number of primary basal dendrites (hSOD1^{G93A}, 4.1 ± 0.1 , $n=5$ mice; WT, 4.3 ± 0.1 , $n=6$ mice; Fig. 4L). Basal dendrites did not exhibit signs of degeneration (Figs. 4I,J, enlarged panels) and basal dendrite spine densities in layer V were comparable (hSOD1^{G93A}, 3.2 ± 0.2 spines/10 μm , $n=5$ mice; WT, 3.4 ± 0.1 spines/10 μm , $n=6$ mice; Fig. 4N).

Cell–cell interactions of CSMN with astrocytes and microglia

We investigated Cell–cell interactions of astrocytes and microglia with WT CSMN soma (n=142 CSMN, n=6 mice), and hSOD1^{G93A} CSMN soma (n=129 CSMN, n=5 mice). CSMN vulnerability was recognized by the presence of pyknotic nuclei as an early sign of apoptosis (Ozdinler et al., 2011; Raoul et al., 2002). Quiescent astrocytes have diffuse and branched cellular morphology different from activated/reactive astrocytes, which exhibit increased GFAP assembly and increases in the number and size of the processes extended from the cell body (Boillee et al., 2006a; Supplementary Fig. 2A). Astrocytes were detected around areas of neuronal activity, such as near soma and in layer II/III (Figs. 5A–F). Quiescent astrocytes were present throughout the motor cortex of the WT mice, and some were in close contact with CSMN soma (80%, n=6; Fig. 5E). However, about 4% of healthy CSMN in the WT mice were in close contact with activated astrocytes. In some rare cases, WT CSMN had pyknotic nuclei (n=34 CSMN, n=6 mice), which were surrounded either by activated astrocytes (13%) or by quiescent astrocytes (64%). In the hSOD1^{G93A} mice there was an increase of CSMN with pyknotic nuclei (62%; Fig. 5F), as previously reported (Ozdinler et al., 2011). However, there was no significant correlation between CSMN health and their cellular contacts with astrocytes near the soma; 21.4% (n=23 CSMN) of CSMN with pyknotic nuclei had no interaction, 21.3% (n=23 CSMN) had Cell–cell interactions with activated astrocytes, whereas 37.4% (n=26 CSMN) had close interaction with quiescent astrocytes. Similarly, no interactions were observed in 16.3% (n=26 CSMN) of healthy CSMN. In contrast, Cell–cell interactions of astrocytes with CSMN apical dendrites showed significant differences between WT and hSOD1^{G93A} mice at P60. About 76±19% of WT CSMN (n=97 apical dendrite, n=6 mice) had close Cell–cell interactions with quiescent astrocytes and only 5% (n=7 CSMN) with activated astrocytes (Figs. 5A,C). However, 43±22% of hSOD1^{G93A} CSMN (n=63 apical dendrite, n=5 mice) apical dendrites were in close contact with quiescent astrocytes, and 40±7% which were notably vacuolated were in direct cellular contact with activated astrocytes (p<0.05; Figs. 5B,D).

CSMN interaction with microglia was examined using cellular markers for macrophage/microglia (e.g. CD11b) and microglia (e.g. Iba-1). Elevated CD11b immunoreactivity in all layers investigated suggests overall increased microglial activation in the hSOD1^{G93A} mice (Figs. 6A–E). In layer IV, hSOD1^{G93A} CSMN apical dendrites were surrounded by microglia with thick, short protrusions and oval nuclei, suggesting an activated state (Philips and Robberecht, 2011; Supplementary Fig. 2B, Figs. 7A–F). Microglia interactions with WT CSMN (n=203 CSMN, n=6 mice) and hSOD1^{G93A} CSMN soma (n=153 CSMN, n=5 mice) revealed higher numbers of CSMN with pyknotic nuclei (n=30 WT CSMN, n=6 mice; n=109 hSOD1^{G93A} CSMN, n=5 mice) to have close cellular contact with activated microglia (17±11% in WT n=6 mice; 55±11% in hSOD1^{G93A} n=5 mice, p<0.05) and a reduction of CSMN interacting with resting microglia (52±11% in WT; 34±6% in hSOD1^{G93A}) (Figs. 7E–F). Only 8±4% of CSMN apical dendrites in WT mice (n=163 apical dendrites, n=6 mice) appeared to interact with activated microglia, and this increased to 53±11% in hSOD1^{G93A} (n=130 apical dendrite; n=5 mice, p<0.05). In addition, CSMN soma interactions with resting microglia were reduced from 66±6% in WT to 30±10% in hSOD1^{G93A} mice (p<0.05; Figs. 7A–D).

Discussion

There are two main limitations in bringing effective cellular therapies to neurodegenerative diseases; one is our lack of understanding for the cellular basis of selective neuronal vulnerability, and the second is the absence of gene delivery approaches targeting only vulnerable neurons within the complex structure of the cerebral cortex. In this study, we overcome both of these limitations. By developing and applying a novel AAV-mediated retrograde transduction approach, which transduces CSMN selectively in both WT and hSOD1^{G93A} transgenic ALS mouse cortex, we reveal important information about CSMN vulnerability at the morphological level and establish gene delivery approaches to CSMN in ALS. Upon eGFP gene transduction, the cytoarchitecture of CSMN was revealed in great detail indicating an early cellular degeneration at P60 in hSOD1^{G93A} mice starting at the apical dendrite, with elimination of spines and pronounced degeneration especially within layer II/III. In addition, astrocytes and microglia were found in close cellular contact with degenerating apical dendrites and soma of hSOD1^{G93A} CSMN, suggesting their importance in CSMN pathology.

Retrograde labeling approaches using fluorescent microspheres or FluoroGold (FG) has led to the identification of CSMN in situ and to their purification and detailed analysis, revealing molecular controls over their differentiation (Arlotta et al., 2005) and responses to growth factors (Ozdinler and Macklis, 2006). However, these applications failed to reveal the full cytoarchitecture of CSMN since microspheres and FG were either encapsulated in lysosomes or were diffusely present within the cell body (Hains et al., 2003; Ozdinler et al., 2011; Shokouhi et al., 2010). In addition, microspheres are unable to carry genetic material to projection neurons, despite their use for protein delivery (Tran et al., 2011).

AAV for retrograde transduction of projection neurons is an emerging idea. Engineering different serotypes using the AAV2 backbone and capsid replacement from other AAVs has led to the generation of numerous serotypes, increasing opportunities for selective transduction (Daya and Berns, 2008). Therefore, identification of the most appropriate serotype offers great advantages as this can both label and transduce the neurons of interest. Retrograde transduction of projection neurons such as nigrostriatal neurons (Eberling et al., 2009), red nucleus (Blits et al., 2010) and spinal motor neurons (Foust et al., 2008, 2009; Kaspar et al., 2003; Snyder et al., 2011) with different AAV serotypes has been reported previously. CSMN have been retrogradely transduced with herpes simplex virus, but with low transduction efficiency (<1%) and lack of detailed cellular morphology (Groutsi et al., 2008).

In this study, we performed a very precise and localized injection of AAV into the CST, thus preventing diffusion into the CSF and blood. Neuronal transduction beyond the site of injection was not apparent, in contrast to other approaches such as intrathecal injections of AAV6 or AAV9, which transduced motor neurons throughout the spinal cord (Snyder et al., 2011). We find AAV2-2 to have a higher level of transduction compared to AAV2-1, AAV2-5, AAV2-7, AAV2-9, and that AAV2-6 and AAV2-8 fail to transduce CSMN using this approach. This difference in transduction efficiency is not likely to be a result of variation in virus delivery to CST axons, as simultaneous labeling with red fluorescent

microspheres revealed comparable CSMN labeling among all serotypes tested. AAV-2 gains entry to target cells by the receptor heparan sulfate proteoglycan, in conjunction with at least six other co-receptors (Daya and Berns, 2008). Capsid protein differences and differences in expression and distribution of appropriate receptors on CSMN axons may both underlie the tissue-specific tropism of various AAV serotypes.

CSMN with their branched apical dendrites and profuse spines, especially within layer II/III, are one of the largest pyramidal projection neurons of the cerebral cortex. Multiple long-range excitatory input pathways, such as corticocortical and thalamocortical projections converge on primary motor cortex and have direct neuronal input to CSMN, through which they modulate their activation and initiation of action potentials (Bureau et al., 2004). Studies in WT mice show that layer II/III is particularly important for CSMN modulation and that CSMN receive disynaptic inhibitory inputs from neurons located in layer V which in turn receive input from pyramidal neurons located in layer II/III (Alstermark and Ogawa, 2004). In addition, CSMN receive long-range excitatory inputs that derive from thalamocortical or corticocortical projection neurons (Anderson et al., 2010). Although it is not yet clear where precise thalamocortical, corticocortical and pre-motor cortex synapses converge on CSMN, it is speculated that one third of CSMN input is modulated via connections in layer II/III, and thus, this layer is remarkably important for CSMN function and activity (Hooks et al., 2011; Weiler et al., 2008).

CSMN output to spinal targets is critical for the initiation and control of voluntary movement and shows differences among species (Striedter, 2006). In humans, motor neuron circuitry from CSMN to SMN is mediated largely by direct connections, whereas in mice, monosynaptic connections with spinal interneurons are predominant (Lemon, 2008). Connectivity differences among species impede modeling of human motor neuron circuitry in mice or any other model organisms. To date, numerous mouse models generated based on known mutations that cause motor neuron diseases in human patients did not show a detectable motor function defect, most likely due to these species differences. One exception has been the hSOD1^{G93A} transgenic ALS mouse model, which mimics many aspects of human pathology. This could be due to the central role of SOD1 in motor neuron health such as oxidative stress and proper mitochondrial function. In addition, since SOD1 is expressed in all cell types in CNS including astrocytes and microglia, mutations in the SOD1 gene may affect cellular functions in these cells, thus contributing to the effects of susceptible motor neurons and leading to complex and non-cell autonomous pathways for neuronal degeneration (Boillee et al., 2006a, 2006b). Both SMN and CSMN vulnerability and progressive degeneration have been characterized in hSOD1^{G93A} mice (Gurney et al., 1994; Ozdinler et al., 2011), but CSMN involvement in disease pathology remains to be elucidated in other mouse models of ALS, such as FUS/TLS (Huang et al., 2011) and TDP-43 (Wegorzewska et al., 2009) lines. We contend that mouse models offer a valuable tool to investigate the cell biology of motor neurons and to understand the cellular and molecular basis of their neuronal degeneration despite differences in connectivity within motor neuron circuitry.

Changes in spine morphology and selective spine degeneration in distinct neuron populations in neurodegenerative diseases are becoming active areas of research.

Morphological changes in neuronal architecture such as dendrite degeneration and spine loss are indicative of defects in neuronal connectivity and sites of neuronal vulnerability. For example, in autism spectrum disorders (ASD) and in schizophrenia, altered synaptic connectivity and reduced plasticity in the brains of patients correlates with disruptions in information processing and cognition (Penzes and Vanleeuwen, 2011; Penzes et al., 2011). Golgi-impregnated post-mortem ASD human brain tissue revealed an increase in spine density on apical dendrites of pyramidal neurons from cortical layer II in frontal, temporal and parietal lobes, and spine density was inversely correlated with cognitive function, as expected (Hutsler and Zhang, 2010). Most interestingly, studies provide strong support for synapse degeneration as having a central role in Alzheimer's disease (AD) pathology. In AD, dendritic spine loss occurs early in the progression of AD and is observed mainly in the hippocampus, suggesting synaptic dysgenesis as a prominent pathology of AD, rather than a byproduct of cognitive decline (DeKosky and Scheff, 1990; Scheff et al., 1990). Neuron type-specific and distinct micro patterns of spine degeneration within the same neuron have intriguing implications for understanding cellular basis of neuronal vulnerability and neuronal connectivity. For example, in schizophrenia spine loss occurs in layer III, but not in layer V or VI (Kolluri et al., 2005). In ASD, increase in spine density is found only in layer II of the frontal and parietal lobe, but no differences are detected in layer V, which suggests layer-specific disruptions in synaptic function of local circuitries as well as neuronal connectivity among other regions of the brain (Hutsler and Zhang, 2010). Likewise, in two different mouse models of AD, spine density reduction is observed specifically in basal proximal dendrites, and not in apical dendrites of the CA1 pyramidal neurons prior to plaque formation, further supporting the importance of selective spine loss as an early event for neuron type-specific vulnerability in neurodegenerative diseases (Perez-Cruz et al., 2011).

Since most of neuronal connectivity is mediated by synapses located on spines, the restricted location of spine loss within a neuron can provide clues on the neuronal circuitries that are primarily affected in disease (Penzes et al., 2011). Here, we observed a profound and selective loss of spines of apical dendrites, especially in layer II/III of the motor cortex in hSOD1^{G93A} mice at an early symptomatic stage. In contrast, basal dendrites did not show signs of degeneration; numbers of primary basal dendrites and their spines were mostly unaffected. Selective degeneration of apical dendrites suggests that hSOD1^{G93A} CSMN are unable to receive proper input to this subcellular site at P60. This may imbalance CSMN excitation/inhibition and eventually lead to vulnerability and cell death. Observations of hyperexcitability reported in patients (Zanette et al., 2002) may be related to this finding and reflect excitotoxic mechanisms leading to the widespread sensorimotor cortex atrophy and degeneration observed especially during end-stage of ALS (Grosskreutz et al., 2006; Nihei et al., 1993), and in part explain why ALS is considered a “systems” degeneration of the cerebral cortex.

The importance of astrocytes and microglia during disease initiation and progression has been studied largely in relation to SMN (Alexianu et al., 2001; Bruijn et al., 2004; Gowing et al., 2008; Hall et al., 1998; Ilieva et al., 2009; Levine et al., 1999). Massive astrogliosis and microgliosis have been detected in motor cortex of ALS patients (Graham et al., 2004; Johansson et al., 2007) and hSOD1^{G93A} mice mainly during end-stage (Ozdinler et al., 2011). However, no detailed cell–cell interaction studies were previously done. In large

projection neurons like CSMN, it is possible that astrocytes and microglia have different modes of actions with basal dendrites, apical dendrites and soma. Especially in the apical dendrites, where spine density is high and neuronal modulation is very prominent, pre- and post-synaptic terminals rely heavily on communication with, and feedback from, surrounding astrocytes, forming a “tripartite synapse” in which synaptic transmission and plasticity are dynamically regulated (Santello et al., 2011). Therefore the presence of quiescent astrocytes suggests their supportive role maintaining synapses and neuronal health. However, in disease, when spines are lost and neuronal connectivity and synapses are hampered, quiescent astrocytes may no longer be needed. As disease progresses, these may be replaced by activated astrocytes and microglia to help clear damaged cellular compartments. In line with this suggested paradigm, astrocytes and microglia may also assume different states of cellular function affecting their supportive roles and contributing to rapid CSMN degeneration due to their expression of mSOD1 (Boillee et al., 2006b). We report that astrocytes and microglia interact mainly with degenerating CSMN apical dendrites and that their interactions with soma do not strongly correlate with the presence of pyknotic nuclei. These observations further support their role in CSMN cellular pathology, but do not suggest their control over initial stages of CSMN vulnerability.

In summary, our findings based on a novel AAV-mediated retrograde transduction approach, reveal an intrinsic mechanism for early stages of CSMN vulnerability and indicate lack of cortical connectivity and circuitry modulation of CSMN as a potential cause of neuronal susceptibility and degeneration in ALS. In addition, our findings offer AAV-mediated retrograde transduction as a tool for future targeted gene therapy approaches to CSMN in the motor cortex, especially valuable for developing therapies that aim to modulate intrinsic factors responsible for CSMN vulnerability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work has been supported by grants from the Les Turner ALS Foundation, Wenske Foundation, Brain Research Foundation (P.H.O), Northwestern University Translational Innovation grant (P.H.O. and M.C.B). J.H.J. was supported by the ALSA Safenowitz postdoctoral fellowship, and S.R.V. by the NIH T32-GM08061 Pre-doctoral training grant. We would like to thank, J. Xie for excellent technical assistance and W. Weber for help with experiments. We would also like to thank B. Goosens from the Microscopy and Imaging Facility at Children's Memorial Research Center for assistance with confocal microscopy. D. Srivastava and P. Penzes helped with initial stages of apical and basal dendrite analysis and generously shared their laboratory resources. NHLBI Gene Therapy Resource Program (GTRP) provided the AAV serotypes.

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Fig. 1.

AAV injections into the corticospinal tract (CST) for retrograde transduction of corticospinal motor neurons (CSMN). (A) Schematic drawings of experimental design depict AAV-eGFP serotype injections at C2–C3 level of the spinal cord into the CST that lies within the dorsal funiculus (df). AAV serotypes and red fluorescent microspheres were injected together into the df of the spinal cord. (B) Schematic drawing of the spinal cord showing the anatomical locations of df, gracile tract (gt), rubrospinal tract (rs) and central canal (cc). (C) Red fluorescent microspheres indicate the location of injection and eGFP confirms minimal AAV transduction within the spinal cord. All injections of WT and hSOD1^{G93A} mice were performed at P30 using a total volume of 0.621 μ l of matched-titer AAV serotypes.

Fig. 2.

CSMN can be retrogradely transduced by AAV2-2, AAV2-5, and AAV2-9, but not AAV2-8, following CST injection. AAV-eGFP matched-titer injections (1.16×10^9 v.p.) into the CST showed varied CSMN transduction levels at P60. Schematic drawings depict experimental design of CSMN retrograde transduction following (A) AAV2-2, (B) AAV2-5, (C) AAV2-8, or (D) AAV2-9 injection into the CST. Retrogradely transduced CSMN express eGFP, contain red fluorescent microspheres, and express CTIP2, confirming CSMN identity. AAV2-2 showed highest retrograde transduction efficiency, whereas AAV2-5 and AAV2-9 showed modest levels. In contrast, AAV2-8 failed to transduce CSMN. Scale bar=50 μ m.

Fig. 3.

AAV2-2 is capable of retrograde transduction of CSMN in the hSOD1^{G93A} transgenic ALS mice. (A) Schematic drawing of experimental design depicts injection of 1.16×10^9 v.p. of AAV2-2 eGFP into the CST of hSOD1^{G93A} mice at P30. (B) Coronal view of cerebral cortex. CSMN are visualized by red fluorescent microspheres, and transduced CSMN are identified by eGFP expression within layer V of motor cortex. (C) Retrogradely transduced CSMN of hSOD1^{G93A} mice contain red fluorescent microspheres and express eGFP and CTIP2, confirming CSMN identity. Scale bar=50 μ m.

Fig. 4.

AAV2-2 eGFP-transduction of CSMN reveals selective apical dendrite degeneration and lack of spines in layer II/III of the hSOD1^{G93A} transgenic ALS mice. (A–J) eGFP-DAB immunocytochemistry following AAV2-2-eGFP transduction reveals precise cellular morphology of CSMN in both WT and hSOD1^{G93A} mice. (A–D) In layer II/III of the motor cortex, WT CSMN are characterized by healthy apical dendrites that branch and arborize and contain spines (B, enlarged images below). In layer IV of the motor cortex, WT CSMN apical dendrites appear healthy (C,D, boxed areas enlarged to the right). (E–H) In contrast, hSOD1^{G93A} CSMN are characterized by lack of spines and a loss of apical dendrites in layer II/III (F, enlarged images below and to the right). Apical dendrites of hSOD1^{G93A} CSMN show vacuolation (G,H, boxed areas enlarged to the right), as well as a stunted morphology (G, arrowhead). (I–J) Cell soma of both WT (I) and hSOD1^{G93A} CSMN (J) in layer V of the motor cortex exhibit similar cellular morphology, with a large pyramidal cell shape, a prominent apical dendrite and basal dendrites. Quantification of CSMN soma area (K), the number of primary basal dendrites (L), and total spines/10 μm at the basal dendrite level (N) did not reveal any defects. Analysis of total spines/10 μm at the apical dendrites (M) and basal dendrite (N) show striking and selective loss of spines only in the apical dendrite of hSOD1^{G93A} mice.

Fig. 5.

AAV2-2-eGFP transduction of CSMN of the hSOD1^{G93A} ALS transgenic mice reveals close cellular interactions with astrocytes in all layers of the motor cortex. (A, B) Immunocytochemical analysis using the astrocyte marker (GFAP; yellow) shows astrocyte and CSMN interactions in both WT and the hSOD1^{G93A} mice. (A) In layer II/III, astrocytes were observed in close proximity to the WT CSMN apical dendrites, and in some cases, these directly interacted with spines. (B) In contrast, three representative images indicate either lack of astrocyte interaction, or reduced cellular contact with the apical dendrites of hSOD1^{G93A} CSMN. (C, D) In layer IV, astrocytes were observed in close proximity to apical dendrites of (C) WT CSMN and (D) hSOD1^{G93A} CSMN. (E, F) In layer V, (E) WT CSMN do not have pyknotic nuclei, and their somas are usually in close proximity to a quiescent appearing astrocyte. (F) hSOD1^{G93A} CSMN interaction with astrocytes varied as depicted by three different examples. However, presence of pyknotic nuclei does not correlate with astrocyte interaction, or astrogliosis. Scale bar=20 μ m.

Fig. 6.

AAV2-2 eGFP-transduction of CSMN of hSOD1^{G93A} transgenic ALS mice reveals close cellular interactions with microglia in all layers of the motor cortex. Immunocytochemical analysis using a microglia/macrophage marker (CD11b; yellow) shows microglia (denoted by *) and CSMN interaction in both WT and hSOD1^{G93A} transgenic ALS mice (boxed areas are enlarged to the side). (A, B) In layer II/III of the motor cortex, microglia are observed in close proximity to the apical dendrites of both (A) WT and (B) hSOD1^{G93A} CSMN. (C–D) In layer IV, microglia with thick processes are evident only in (D) hSOD1^{G93A} transgenic ALS mice, and these primarily interact with apical dendrites containing vacuoles. (E, F) Microglia are in close proximity to both (E) WT and (F) hSOD1^{G93A} CSMN soma. Microglia partially engulfing CSMN that have pyknotic nuclei were observed (arrow). Scale bar=20 μm.

Fig. 7.

AAV2–2 eGFP-transduction of CSMN of hSOD1^{G93A} transgenic ALS mice reveals close cellular interactions with microglia in all layers of the motor cortex. Immunocytochemical analysis using a microglia marker (Iba-1; yellow) shows microglia (denoted by *) and CSMN interaction in both WT and hSOD1^{G93A} transgenic ALS mice (boxed areas are enlarged to the side). (A, B) In layer II/III of the motor cortex, microglia are observed in close proximity to the apical dendrites of both (A) WT and (B) hSOD1^{G93A} CSMN. (C, D) In layer IV, microglia with thick processes are evident only in (D) hSOD1^{G93A} transgenic ALS mice, and these primarily interact with apical dendrites containing vacuoles. (E, F) Microglia are in close proximity to both (E) WT and (F) hSOD1^{G93A} CSMN soma. Microglia partially engulfing CSMN that have pyknotic nuclei were observed (arrow). Scale bar=50 μm (A–F); scale bar=20 μm for enlarged inserts.