Downregulation of the Potassium Chloride Cotransporter KCC2 in Vulnerable Motoneurons in the SOD1-G93A Mouse Model of Amyotrophic Lateral Sclerosis

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Abstract
The balance between excitatory and inhibitory synaptic inputs is critical for the physiological control of motoneurons. The maintenance of a low-intracellular chloride concentration by the potassium–sodium cotransporter 2 (KCC2) is essential for the efficacy of fast synaptic inhibition of mature motoneurons in response to the activation of ionotropic γ-aminobutyric acid A and glycine receptors. Altered synaptic balance and excitotoxicity have been proposed as candidate pathophysiological processes in amyotrophic lateral sclerosis (ALS). Therefore, we investigated the expression patterns of candidate pathophysiological processes in amyotrophic lateral sclerosis (ALS). Therefore, we investigated the expression patterns of KCC2 and its functional opponent, the chloride influx–mediating sodium–potassium chloride cotransporter 1 (NKCC1), in the superoxide dismutase 1 (SOD1-G93A) mouse model of ALS. We detected reduced KCC2 messenger RNA levels and less membrane-bound KCC2 immunoreactivity in ALS-vulnerable motoneurons in lumbar spinal cord and hypoglossal nuclei of SOD1-G93A mice but not in degeneration-resistant oculomotor nuclei. Downregulation of KCC2 started during late presymptomatic stages and accelerated in parallel to hind limb and tongue motor function deficits. In contrast, NKCC1 messenger RNA levels were unaltered in postnatal lumbar spinal cord motoneurons. Our data indicate that reductions in KCC2 gene expression may contribute to selective motor deficits and disease progression in vulnerable motoneurons in a mouse model of ALS.

Key Words: Amyotrophic lateral sclerosis, Hypoglossal nucleus, KCC2, Motoneuron, Neurodegeneration, NKCC1.

INTRODUCTION
Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by a specific and progressive loss of large-size upper and lower motoneurons (1). Whereas most (~90%) ALS cases occur with unknown etiology (sporadic), approximately 10% are inherited (familial). Excitotoxic damage of motoneurons is a possible mechanistic link between sporadic and familial ALS (2). On one hand, dysregulated synaptic release and clearing of the excitatory neurotransmitter glutamate is thought to lead to an excessive influx of calcium ions through calcium-permeable ionotropic glutamate receptors, resulting in an elevation of intracellular calcium to toxic levels that ultimately damage and kill the neuron (3). Conversely, altered motoneuron excitability due to abnormal membrane properties may also affect the disease pathogenesis (4, 5).

Motoneurons receive excitatory glutamatergic input and are under inhibitory control by γ-aminobutyric acid A (GABA)-ergic and glycinergic synaptic input (6). Both GABA and glycine bind to ligand-gated ionotropic receptors on the postsynaptic membrane, which results in the opening of an anion-permeable channel. Because the intracellular concentration of chloride ions ([Cl\text{\textsuperscript{-}}]) is low compared with the extracellular milieu and the chloride equilibrium potential (E\text{\textsubscript{Cl}}) is more negative than the membrane potential at rest (V\text{\textsubscript{m}}), there is a net influx of chloride ions along its electrochemical gradient. This shifts V\text{\textsubscript{m}} to more negative values, that is, it hyperpolarizes the membrane potential and renders the neuron less excitable.

Effective chloride extrusion mechanisms are necessary to maintain the steep chloride gradient in mature neurons. In mammals, a family of plasmalemmal ion transporters, the cation chloride cotransporters (CCCs), plays a crucial role in regulating the strength and polarity of GABAergic and glycinergic neurotransmission (7, 8). Expression pattern analysis has revealed that members of the CCC gene family (also called solute carrier family 12, Slc12) are expressed in neurons and/or glial cells during some phases of central nervous system (CNS) development (9). The 2 CCC family members, the potassium chloride cotransporter 2 (KCC2) and the sodium–potassium chloride cotransporter 1 (NKCC1), are the dominant CCCs in neurons. The expression of KCC2 is restricted to CNS neurons (10). The KCC2 extrudes Cl\text{\textsuperscript{-}} in 1:1 stoichiometry with K\textsuperscript{+} from the cell (11). On the other hand, NKCC1 is expressed ubiquitously in neurons and glial cells (12) and mediates Cl\text{\textsuperscript{-}} influx in 2:2 stoichiometry with Na\textsuperscript{+} and K\textsuperscript{+} (13). In the immature nervous system, high levels of NKCC1 in some neurons are considered responsible for the excitatory actions of GABA and glycine, by keeping...
[Cl\(^-\)]_i high. A developmental switch from depolarizing to hyperpolarizing actions of GABA and glycine is due to a downregulation of NKCC1 and a concurrent upregulation of KCC2 (14, 15). Spinal motoneurons are among the first CNS neurons that switch during development (9), and adult motoneurons have low amounts of NKCC1 messenger RNA (mRNA) while expressing high levels of KCC2 mRNA (12).

Downregulation of KCC2 or upregulation of NKCC1 results in overexcitability and/or excitotoxicity and is associated with diseases such as temporal lobe epilepsy, neurogenic pain, and spasticity after spinal cord lesion (16, 17). We hypothesized that such a mechanism may also be present in ALS and thus examined tissue sections from SOD1-G93A transgenic (Tg) mice (18), the most frequently used mouse model of ALS, for KCC2 and NKCC1 expression patterns by in situ hybridization (ISH) and immunohistochemistry (IHC). We specifically investigated KCC2 and NKCC1 mRNA and protein to determine whether they are differentially regulated and expressed in ALS-resistant versus ALS-vulnerable motor neuron pools in brain stem and spinal cord during disease progression.

MATERIALS AND METHODS

Animals

Transgenic mice of the strain B6SJL-TgN(SOD1-G93A)1Gur (The Jackson Laboratory, Bar Harbor, ME), which carry human SOD1 with the pathogenic G93A mutation (SOD1-G93A) in high copy number, and wild-type (WT) littermates were used. Male and female progeny for experimental analysis were obtained from repeated breeding of SOD1-G93A males with B6SJL/F1/J WT females.

Transgenic mice were identified by polymerase chain reaction on genomic DNA obtained from ear punches using established primers and protocols (for details, see http://jaxmice.jax.org/strain/0027276.html). All mice were housed in groups of 2 to 4 animals per cage on a 12-hour dark-light cycle, 21°C, and 50% to 60% humidity with unrestricted access to food and water. From the time Tg mice showed motor deficits, additional moisturized food and water were placed on the cage floor. Because of ethical considerations, end-stage Tg animals were killed when their weight dropped below 80% of the weight at postnatal day (P) 49 or when they were unable to right themselves when placed on their sides. All animal procedures were performed according to the German Animal Protection Law under a protocol approved by the county administrative government in Giessen (Hesse, Germany), and adequate measures were taken to minimize pain or discomfort.

Motor Function and Disease Progression

Starting at P49, motor performances of SOD1-G93A and WT mice of both sexes were assessed weekly with 2 tests. Motor abilities of the hind legs were assessed with the hind-paw grip endurance test (19). Mice were placed individually on a meshed wire platform at a height of approximately 60 cm above the bench. After the animals found a grip, the platform was gently turned upside-down, and the latency was recorded until the animals disengaged both hind legs from the mesh unless they succeeded to reach the 120-second cutoff time. Each mouse was given a maximum of 3 consecutive trials to reach the cutoff, and the longest latency was recorded. The motor performance of the tongue was determined by automatically recording licking events according to a recently published protocol (20). The test cage was equipped with a metal floor plate and a drinking bottle with a metal sipper tube. The drinking bottle contained 3.5% (vol/vol) fat milk supplemented with 5% (wt/vol) sugar. Using an analog/digital converter (USB6008; National Instruments, Austin, TX), licking-induced junction potentials were recorded when the mice touched the metal sipper and analyzed with a computer equipped with NI-DAQMX v 1.60 software and a LabVIEW-Runtime 7.1.1 Engine (National Instruments). Mice were fluid-restricted overnight before testing. The licking frequency was determined by averaging time intervals between 25 peaks from continuous licking periods of at least 5 seconds. As additional parameters for disease progression, the body weight (BW) was determined weekly, and the survival of the SOD1-G93A mice was monitored.

Tissue Processing

Female and male mice were killed by inhalation of an overdose isoflurane (Baxter, Unterschleissheim, Germany). Tissues were processed in 2 different ways: For ISH experiments, the lumbar spinal cord and the brain were dissected, submerged, and oriented in TissueTek (Sakura Finetic, Zoeterwoude, Netherlands), frozen by immersion in isopentane cooled to −40°C and stored at −70°C until further use. Serial frozen sections 20 μm thick were cut with a cryostat (CM3050S; Leica, Wetzlar, Germany) and mounted on silanized glass slides.

For IHC analysis, the lumbar spinal cord and the brain were dissected and fixed in Bouin Holland solution, containing 4% (wt/vol) picric acid, 2.5% (wt/vol) cupric acetate, 3.7% (vol/vol) formaldehyde, and 1% (vol/vol) glacial acetic acid. After fixation, the tissues were extensively washed in 70% isopropanol, dehydrated, cleared with xylene, and embedded in paraffin. Serial sections 7 μm thick were cut with a microtome (Microm HM325; Thermo Fisher Scientific, Walldorf, Germany) and mounted onto silanized glass slides. Counterstaining was done with Giemsa or cresyl violet solutions.

Generation of Riboprobes, Radioactive, and Nonradioactive ISH

A vesicular acetylcholine transporter (VACht) riboprobe has been published previously (21). Complementary RNA probes for the detection of mouse NKCC1 and KCC2 transcripts in tissue sections (22) were generated from mouse C57BL/6 spinal cord complementary DNA. For NKCC1, a 600-nucleotide-long DNA fragment (GenBank accession no. NM_009194, bp 616–1215), and for KCC2, a 494-nucleotide-long DNA fragment (GenBank accession no. NM_020333, bp 273–775) were amplified by polymerase chain reaction, subcloned into pGEM-T (Promega, Mannheim, Germany). The sequences were confirmed by double-stranded sequencing. After linearization with appropriate restriction enzymes, 35S-labeled riboprobes were generated using either T7 or SP6 RNA polymerase, and the ISH procedure was essentially performed as previously described (23).
The detection of 2 different RNA transcripts on the same tissue section was performed with radioactive and non-radioactive-labeled probes as previously published (24). A digoxigenin (Dig)-labeled VAChT riboprobe was generated by in vitro transcription with a Dig-labeling mix containing 10 mmol/L each of ATP, CTP, and GTP; 6.5 mmol/L UTP; and 3.5 mmol/L Dig-11-UTP (Roche, Basel, Switzerland). The VAChT-Dig riboprobe was added at a concentration of 2 ng/μL to the 35S-labeled NKCC1 and KCC2 hybridization mixes, respectively. Hybridization and posthybridization washes were performed as in Schutz et al (23), except for the final submersion of the slides in isopropanol. For the detection of nonradioactive hybrids, IHC using alkaline phosphatase–conjugated anti-Dig Fab fragments was performed according to the manufacturer’s instruction (Dig-Nucleic Acid Detection Kit; Roche). For the detection of the 35S-labeled riboprobes, slides were covered with K5 photoemulsion (Ilford, Marly, Switzerland) diluted 1:1 in water. After exposure for 21 days in the dark at 4°C, slides were developed and embedded with 37°C warm gelatin embedding medium. Bright and dark field microscopic analysis was performed using an Olympus AX70 microscope (Olympus Optical, Hamburg, Germany).

For semiquantitative analysis of ISH experiments, the 35S-labeled sections were viewed under dark field illumination, live-digitized with a SPOT camera (Diagnostics Instruments, Seoul, Korea), and analyzed with MCID software (MCID Elite 7.0; Imaging Research, St Catharines, Ontario, Canada). After setting a constant detection threshold and an optical plane to view silver grains in a single focal plane, whole motor nuclei were marked free-hand (=total area) and the area covered by silver grains (=labeled area) was measured. Finally, the total–labeled area ratio was calculated. This

FIGURE 1. Assessment of disease progression in SOD1-G93A mice. (A, B) Time course of body weight changes in female (A) and male (B) wild-type (WT, n = 13 mice/sex) and SOD1-G93A (n = 5–10 mice/sex) mice. (C) Time course of motor performance in the hind-paw grip endurance test (n = 26 WT and 15 SOD1-G93A mice). (D) Time course of lick frequency changes (n = 19–26 genotype/age). Data represent mean ± SEM: *p < 0.05; **p < 0.01; ***p < 0.001 (vs WT; 3-way analysis of variance followed by Bonferroni t test for body weight and lick frequency, Mann-Whitney U test separately for each time point for hind-paw grip endurance test); **p < 0.01 (compared with P49; 1-way analysis of variance followed by Dunnett t test).
method was used to determine motoneuron loss in defined neuronal populations. In addition, single motoneuron profiles were marked, and their silver grain density was measured to compare KCC2 expression levels in individual motoneurons between genotypes of the same age.

**Immunohistochemistry**

Immunohistochemistry was performed on deparaffinized tissue sections (7 μm) from different brainstem regions and lumbar spinal cord using an antigen retrieval technique and visualized enzymatically with 3,3′-diaminobenzidine (Sigma, Deisenhofen, Germany) and enhanced by addition of ammonium nickel sulfate (Fluka, Buchs, Switzerland) or (Sigma, Deisenhofen, Germany) and visualized by immunofluorescence as described (21). The antibodies used were rabbit anti-KCC2 (diluted 1:1000 for enzyme-based detection and 1:100 for fluorescent detection; catalog no. 07-432; Upstate/Biomol, Hamburg, Germany) and 1:100 for fluorescent detection; visualized by immunofluorescence as described (21). The antibodies used were rabbit anti-KCC2 (diluted 1:1000 for enzyme-based detection and 1:100 for fluorescent detection; catalog no. 07-432; Upstate/Biomol, Hamburg, Germany) and goat anti–choline acetyltransferase (ChAT; diluted 1:1000; catalog no. AB144P; Chemicon Europe, Hofheim, Germany). Serial immunostaining was carried out on adjacent sections. IHC signals were analyzed and documented with an Olympus AX70 microscope or with an Olympus Fluoview confocal laser scanning microscope (Olympus Optical).

**Statistical Analysis**

For quantification of relative grain area in motor nuclei of single hemispheres of ISH experiments, 1-way analysis of variance (ANOVA) followed by Bonferroni t test was used. For the quantification of relative grain area in single neurons of ISH experiments, a Mann-Whitney U test was used to detect differences between genotypes of same age. Changes in BW, grip strength, and licking were analyzed by 3-way ANOVA (sex, age, and genotype) followed by Bonferroni t test (comparison to WT) and by 1-way ANOVA separately for genotypes followed by Dunnett test (comparison to P49). Hind-paw grip endurance data were subjected to Mann-Whitney U test, separately for each time point.

**RESULTS**

**Assessment of Disease Progression in SOD1-G93A Mice**

SOD1-G93A mice increasingly lose BW and motor functions during disease progression after a presymptomatic phase until approximately P80 (19). Thus, we recorded the BWs of female and male WT and SOD1-G93A mice on a weekly basis and observed a sex difference with respect to onset of signs (Fig. 1). In WT mice, there was a steady increase in BW throughout the examination period (Figs. 1A, B). The BWs of female SOD1-G93A mice were generally lower than their WT littermates, but this difference became significant only from P98 onward (Fig. 1A), after reaching a plateau of maximum weight. Male SOD1-G93A mice, on the other hand, had reached their maximum BW of approximately 26 g already at the beginning of the examination period, which they maintained until P112 (Fig. 1B). Disease-related BW decline was observed at P119. Hind-paw grip endurance tests were performed to assess changes in motor abilities. There was a sex-independent onset of hind leg motor dysfunction in the SOD1-G93A mice at P91 with this test (Fig. 1C). To evaluate muscular atrophy and dysfunction associated with degeneration of bulbar motoneurons, we used a licking test to assay the hypoglossal motor function (Fig. 1D). In WT mice, we measured a mean licking frequency of 10.32 ± 0.1 Hz (mean of P49–P133 ± SEM) in both sexes, which did not change during aging. However, in SOD1-G93A mice, there was an initial phase of stable but already significantly (p < 0.01) reduced licking frequency compared with WT with 9.12 ± 0.12 Hz (mean of P77–P119 ± SEM) between P77 and P119. A second phase of progressive licking frequency reduction began after P119, ending with 7.81 ± 0.93 Hz (mean ± SEM, p < 0.001) at P133.

In female SOD1-G93A mice, there was a correlation between the appearance of lumbar motor dysfunction and reduction in body mass (i.e. muscle tissue) at around P90. In male SOD1-G93A mice, however, reductions in hind limb motor function preceded overt changes in body mass by 3 to 4 weeks. In contrast to BW changes, motor disability appearance and progression were sex-independent. Reductions in the frequency of tongue protrusions during licking showed a biphasic progression with an initial drop at P77, a subsequent stable phase until P119, and a second disease end-stage–related progressive loss of motor abilities at P126.

**Age-Related Changes in NKCC1 Transcript Patterns in the Lumbar Spinal Cord Are Not Associated With Motoneurons or Disease Progression**

Murine spinal motoneurons show minimal expression of NKCC1 mRNA and immunoreactivity (ir) during postnatal life (12, 25), but pathologic upregulation of neuronal NKCC1 mRNA has been associated with temporal lobe epilepsy (26). To determine whether such changes occur in ALS mice, we analyzed the NKCC1 mRNA expression profile in the lumbar spinal cord of WT and SOD1-G93A mice by ISH from P10 to P120 (Fig. 2). At P10, NKCC1 transcripts were mainly localized to cells in the white matter, both in WT (Fig. 2A) and SOD1-G93A (Fig. 2B) mice. In addition, some cells (neurons and/or glia) in the dorsal and the ventral gray matter were ISH+. Absence of NKCC1 ISH signals over the large-sized neuronal profiles in the ventral horn gray matter indicated low to absent expression in motoneurons. At P80, the expression in the white matter had largely disappeared, and signals in the gray matter were found over numerous, mainly small-sized cells; there were no obvious difference between

**FIGURE 2.** Sodium–potassium chloride cotransporter 1 (NKCC1) mRNA expression profile in the lumbar spinal cord. (A–F) Representative dark field images from in situ hybridization experiments with a NKCC1 riboprobe at postnatal day (P) 10 (A, B), P80 (C, D), and P120 (E, F) from wild-type (WT) and SOD1-G93A mice. (G, H) Mixed dark and bright field images from double in situ hybridization experiments with 35S-labeled NKCC1 riboprobes (white dots) and digoxigenin-labeled vesicular acetylcholine transporter (VACHT) riboprobes (dark reaction products). Scale bars = (A) 500 μm for A to F; (G) 25 μm and for panel H.
WT (Fig. 2C) and SOD1-G93A (Fig. 2D) mice. This pattern remained unchanged until P120 (Figs. 2E, F). Double ISH experiments were performed to evaluate whether NKCC1 transcripts were upregulated in motoneurons during disease progression in SOD1-G93A mice versus age-matched WT mice, but that was not the case at P120 (Figs. 2G, H). Only a few NKCC1 transcript ISH signals were detectable over VACHT+ motoneurons in WT (Fig. 2G) and SOD1-G93A (Fig. 2H) mice. On the basis of these findings and on similar findings in other motor neuron pools (e.g. cranial nerve motor

**FIGURE 3.** Potassium chloride cotransporter 2 (KCC2) mRNA expression profile in the lumbar spinal cord. (A-L) Dark field images from in situ hybridization (ISH) experiments with a KCC2 riboprobe in wild-type (WT) and SOD1-G93A transgenic mice at 3 representative ages. Low-magnification images (A, C, E, G, I, K) illustrate KCC2 mRNA distribution on whole transverse sections; high-magnification images (B, D, F, H, J, L) are from the respective motor neuron areas. Arrows point to strong labeling of motoneurons. Double arrows mark motoneurons with reduced KCC2 ISH signals. (M, N) Bright field images from double-ISH experiments with 35S-labeled KCC2 riboprobes (black dots) and digoxigenin-labeled vesicular acetylcholine transporter (VACHT) riboprobes (dark reaction product) taken from WT (M) and SOD1-G93A (N) mice. (O) Scatter plot of semiquantitative evaluation of KCC2 mRNA abundance measuring relative grain area over single motoneurons. All values (n = 14–25 neurons from 4 mice/genotype/age) plus mean (black horizontal line). *p < 0.01 (Mann-Whitney U test). Scale bars = (A) 500 µm and for C, E, G, I, K; (B) 50 µm and for D, F, H, J, L; (M) 25 µm and for N.
nuclei [data not shown]), we did not do further evaluation of NKCC1 expression patterns.

**KCC2 Transcripts Are Downregulated in ALS-Vulnerable Motoneurons During Disease Progression**

The expression of KCC2 is restricted to neurons, and that of KCC2 mRNA is strong in adult spinal and bulbar motoneurons (12). Because KCC2 mRNA is downregulated in injured motoneurons leading to overexcitability (27), we analyzed KCC2 mRNA in the 2 motoneuron pools that showed disease-related motor dysfunction during functional testing, that is, those in the lumbar spinal cord and the hypoglossal nucleus. In situ hybridization signals for KCC2 were restricted to lumbar spinal cord gray matter and were absent from the white matter (Fig. 3). At P10, the neurons of the lumbar spinal cord gray matter were densely packed, and all showed strong KCC2 ISH signals, both in WT (Fig. 3A) and SOD1-G93A (Fig. 3C) mice, including large motoneurons (Figs. 3B, D). The expression pattern of KCC2 remained unchanged throughout age in WT mice (Figs. 3E, F I, J). In SOD1-G93A mice, however, some large neurons in the ventral horn displayed reduced KCC2 transcript intensities at the late preclinical age of P80 (Figs. 3G, H), whereas other regions of the spinal cord were largely unaffected at this age. At disease end-stage (P120), only a few, mainly smaller neurons exhibited WT-like KCC2 ISH signals, whereas most of the remaining large motoneurons displayed reduced KCC2 signal intensities (Figs. 3K, L). This downregulation of KCC2 message in motoneurons was confirmed qualitatively in double ISH experiments. Whereas all WT and few SOD1-G93A motoneurons stained strongly for KCC2 ISH signals (Figs. 3M, N), some motoneurons from SOD1-G93A mice displayed fewer ISH signals (Fig. 3N). Quantification of KCC2 ISH signals over single motoneurons revealed no difference between WT (0.27 ± 0.01, mean ± SEM) and SOD1-G93A mice (0.25 ± 0.01, mean ± SEM, not significant [NS]) at P80, but a significant downregulation of KCC2 message in SOD1-G93A mice (0.22 ± 0.01, mean ± SEM) versus WT (0.26 ± 0.01, mean ± SEM, p = 0.0061) mice at P120 (Fig. 3O).

The hypoglossal nucleus also degenerates in SOD1-G93A mice, as indicated by a cell loss of 12% at P80 to P90, a 28% loss by P130 to P140 (28), and massive vacuolization (29). We assessed KCC2 transcript abundance in this nucleus with ISH and compared them with the mRNA expression profile of VACHT, a marker of cholinergic neurons (30). All

![FIGURE 4. Vesicular acetylcholine transporter (VACHT) and potassium chloride cotransporter 2 (KCC2) mRNA expression in the hypoglossal nucleus. (A–D)](https://jnen.oxfordjournals.org/content/69/10/1063/F4) Dark field images from in situ hybridization experiments with VACHT (A, C) and KCC2 (B, D) riboprobes on adjacent transverse sections from P120 wild-type (WT) and SOD1-G93A mice. Arrows in (D) point to motoneurons with reduced KCC2 mRNA grain densities. X indicates dorsal motor nucleus of the vagus nerve; XII, hypoglossal nucleus. (E) Scatter plot of KCC2 relative grain area in single hypoglossal motoneurons (n = 60 values from 4 mice/genotype/age). *p < 0.05; **p < 0.01 (gray horizontal line = mean, Mann-Whitney U test). Scale bar = (A) 200 μm and for A to D.)
**FIGURE 5.** Potassium chloride cotransporter 2 (KCC2) immunoreactivity (ir) patterns in the lumbar spinal cord. **(A–D)** Bright field images from transverse sections stained for KCC2-ir in wild-type (WT) and SOD1-G93A mice. **(E, F)** Higher magnifications of the motoneuron area (indicated by arrows in D), showing KCC2-ir in the neuropil and absence of KCC2-ir in motoneuron cytoplasm (arrow in E) in a WT mouse, and reductions in KCC2-ir in the neuropil but appearance of KCC2-ir in motoneuron cytoplasm (arrows in F) in a SOD1-G93A mouse. Scale bars = (A) 500 μm and for A to D; (F) 50 μm and for E and F.
cholinergic motoneurons of the hypoglossal nucleus and the adjacent parasympathetic dorsal nucleus of the vagus nerve displayed VAChT ISH signals with similar intensities in WT (Fig. 4A) and SOD1-G93A mice (Fig. 4C) at P120. The KCC2 ISH signals were less intense than those of the VAChT but were equally strong in all motoneurons in WT mice (Fig. 4B). Hypoglossal neurons in SOD1-G93A mice had reduced KCC2 ISH signal intensities (Fig. 4D, arrows). Relative quantification of KCC2 mRNA levels in single neurons (Fig. 4E) revealed downregulation of KCC2 mRNA already at P80 in SOD1-G93A mice (0.4 ± 0.1, mean ± SEM) vs. WT (0.46 ± 0.09, mean ± SEM, p = 0.002), which persisted at P120 (0.46 ± 0.12 for WT vs. 0.41 ± 0.1 for SOD1-G93A, p = 0.019; Fig. 4E). The narrow distribution of many individual values around the mean (Fig. 4E) indicates that reductions in KCC2 mRNA occurred in many neurons rather than that there were large reductions in a small number of highly affected neurons, suggesting that KCC2 loss is important in the pathogenesis at the onset of functional impairment (compared with Fig. 1D).

**KCC2 Immunoreactivity Is Reduced in the Neuropil and Enhanced in Motoneuron Cytoplasm of SOD1-G93A Mice**

The distribution of KCC2 protein in the lumbar spinal cord was determined by IHC. At P80, both in WT (Fig. 5A) and in SOD1-G93A (Fig. 5B) mice, prominent KCC2-ir was observed predominantly on neurites and membranes of cell soma throughout the gray matter, with some KCC2-ir neuronal processes also extending into the surrounding white matter. Neuronal cytoplasm was largely free of KCC2-ir. There were no obvious differences in staining patterns between WT and SOD1-G93A mice. At P120, however, this pattern was preserved in WT mice (Fig. 5C), but SOD1-G93A mice showed reductions in KCC2-ir that were not restricted to but were most prominent in the motoneuron area of the lateral ventral horn (Fig. 5D). There were reductions in KCC2-ir in the neuropil surrounding motoneurons (Fig. 5F), whereas some motoneuron cell bodies had stronger cytoplasmic KCC2-ir (Fig. 5F) than motoneurons in WT mice (Fig. 5E). These findings suggest that there is a disease-related internalization of KCC2 in long-surviving spinal motoneurons in the SOD1-G93A mice.

Similar strong KCC2-ir covered the entire hypoglossal nucleus at P40 in WT (data not shown) and SOD1-G93A mice (Fig. 6B), sparing ChAT-ir neuronal cell bodies (Fig. 6A), another marker for cholinergic neurons. At P80 in SOD1-G93A mice, there was a reduced intensity of KCC2-ir mainly in the ventral part where the most vulnerable gigantosomatal subnucleus of the hypoglossal nucleus is located (Fig. 6D). This region still contained many motoneurons, however, as indicated by ChAT-ir on an adjacent section (Fig. 6C). At later time points (including P120), reductions in KCC2-ir spread over the entire nucleus (Fig. 6F), whereas numerous ChAT-ir neurons were still present (Fig. 6E). Reductions in KCC2-ir throughout disease progression were also evident in the dorsal nucleus of the vagus nerve (Figs. 6D, F). As in the lumbar spinal cord, the hypoglossal motoneurons showed subcellular redistribution of KCC2-ir. A faint band of KCC2-ir was seen at the somatic plasma membrane in WT mice (Fig. 7A), whereas KCC2-ir was found more dispersed in the cytoplasm and largely absent from the cell surface in SOD1-G93A mice (Fig. 7B).

**KCC2 Expression Is Unaltered in the ALS-Resistant Oculomotor Nucleus**

To determine whether reductions in KCC2 expression spared ALS-resistant motoneurons, we analyzed the oculomotor nucleus, a motoneuron pool that is largely unaffected by neurodegeneration in ALS (28). Strong ISH signals for VAChT mRNA were detectable in the cholinergic oculomotor neurons both in WT (Figs. 8A, E) and throughout disease progression in SOD1-G93A mice (Figs. 8C, G). Quantification of the grain area of VAChT ISH as a measure for motoneuron loss revealed a tendency toward an age-related decline in the number of motoneurons both in WT and SOD1-G93A mice between P40 and P120 (Fig. 8I) (WT: 0.29 ± 0.08 at P40 vs. 0.24 ± 0.09 at P80 vs. 0.16 ± 0.05 at P120, n = 3 mice, NS; SOD1: 0.22 ± 0.11 at P40 vs. 0.12 ± 0.10 at P80 vs. 0.16 ± 0.07 at P120, n = 3 mice, NS). In situ hybridization signals for KCC2 mRNA were less intense compared with those for VAChT mRNA, but the probes labeled motoneurons equally in WT (Figs. 8B, F) and in SOD1-G93A mice (Figs. 8D, H). As for VAChT mRNA, there was only a tendency toward a reduction in the number (Fig. 8J) or in the level of KCC2 message in oculomotor neurons until disease end-stage (WT: 0.11 ± 0.07 at P40 vs. 0.04 ± 0.03 at P80 vs. 0.04 ± 0.03 at P120, n = 3 mice, NS; SOD1: 0.10 ± 0.05 at P40 vs. 0.08 ± 0.08 at P80 vs. 0.04 ± 0.03 at P120, n = 3 mice, NS). Our observations on the transcript level were confirmed on the protein level by IHC. Similar staining patterns were detected at P120 in WT (Fig. 8K) and SOD1-G93A mice using ChAT-ir (Fig. 8M). There was strong KCC2-ir of fibers in the oculomotor nucleus and adjacent brain areas, both in WT (Fig. 8L) and in SOD1-G93A mice (Fig. 8N). Similar to the motoneurons in the WT mice lumbar spinal cord, KCC2-ir was restricted to neurites and largely absent from the cytoplasm in both genotypes (insets in Figs. 8L, N), indicating absence of a disease-related intracellular KCC2 redistribution.

**DISCUSSION**

The major findings of this study are as follows: 1) KCC2 mRNA levels in ALS-vulnerable motoneurons in SOD1-G93A mice are downregulated compared with WT mice, but they are preserved in ALS-resistant motoneurons; 2) KCC2-ir is reduced in neurites surrounding vulnerable motoneurons but is enhanced in the cytoplasm of motoneuron cell bodies at the expense of plasmalemmal localization; 3) changes in KCC2 expression patterns parallel motor function deficits; and 4) NKCC1 mRNA levels in spinal motor neurons in SOD1-G93A mice do not differ from those in WT mice during postnatal life. Although they are primarily inhibitory for intact adult motoneurons, both GABAergic and glycinergic receptors during exposure to pathologically elevated glutamate levels may result in increased Ca²⁺ influx through α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors and/or...
Activity of KCC2 is regulated not only at the level of transcription but also by subcellular targeting (22) and post-translational modification (8). In WT mice, we found that KCC2-ir localized to the membrane of the cell soma and to dendrites. In SOD1-G93A mice, however, KCC2-ir was reduced in neurites surrounding motoneurons and enhanced in the cytoplasm at the expense of plasmalemmal localization. Similar changes in subcellular distribution were also observed in lumbar motoneurons after thoracic spinal cord injury (17). The loss of KCC2 from dendrites and the subcellular mislocalization might thus challenge the Cl\textsuperscript{−}-extruding capacity of a neuron and promote cell damage.

The function of KCC2 is regulated by posttranslational phosphorylation and dephosphorylation. Phosphorylation at serine-threonine residues is thought to inhibit KCC2 and may also be activated by phosphorylation of tyrosine residues (35), resulting in increased plasmalemmal stability of the phosphorylated protein (36). Inasmuch as human ALS-vulnerable motoneurons shrink (37), and activation of cell volume-sensitive serine-threonine kinases is thought to inhibit KCC2 and may be subsequent phosphorylation of KCC2. Similarly, oxidative stress and overexcitation may result in dephosphorylation of KCC2 tyrosine residues (38). Both scenarios would provide evidence for an alternative mechanism of excitotoxicity that involves disinhibition of motoneurons. The KCC2 is a crucial factor for synaptic inhibition of motoneurons because the absence of KCC2 results in an excitatory action of GABA and glycine. Moreover, KCC2\textsuperscript{−/−} mice die immediately after birth because of severe motor deficits (22). In contrast to such a dramatic situation (and more relevant for most neurologic diseases), even a subtle downregulation of KCC2 may have profound consequences on the way intracellular Cl\textsuperscript{−} is handled by motoneurons; such perturbations may influence the strength and polarity of GABAergic and glycineric neurotransmission (32). As long as the Cl\textsuperscript{−} extrusion capacity of the motoneuron is kept high because of the normal expression and activity of KCC2, low intracellular Cl\textsuperscript{−} concentration is maintained. As a consequence, the reversal potential for GABA ($E_{GABA}$) is close to or even below the neurons resting membrane potential of approximately $\sim$63 mV (32). GABAergic and glycineric synaptic input then triggers Cl\textsuperscript{−} influx and hyperpolarization. On the contrary, a low Cl\textsuperscript{−} extrusion capacity due to a reduction in KCC2 expression and a subsequent change in $E_{GABA}$ may trigger Cl\textsuperscript{−} efflux and depolarization, thus increasing the excitability of motoneurons (33). In a rat model of spinal cord injury (17), a decrease of 10% to 20% in KCC2 protein content (similar to the results of this study) induced an approximately 10 mV depolarizing shift in $E_{GABA}$, mediated inhibitory postsynaptic potentials. Such a depolarizing shift might have a functional effect on GABA\textsubscript{A}, and glycine receptor responses (triggering action potentials instead of inhibiting them) depend on the degree of shift (33); the greater the upward shift in $E_{GABA}$, the more likely there will be a net excitatory action. Moreover, even small depolarizing inhibitory postsynaptic potentials may facilitate glutamatergic input, so that subthreshold excitations reach the action potential threshold (17). The limited effect of riluzole in the treatment of ALS and the prominent reductions in the numbers of glutamatergic synapses on spinal cord motoneurons during disease progression suggest that glutamatergic excitotoxicity may play only a minor role in the disease pathogenesis (34). Nevertheless, an interplay of pathways regulating and responding to changes in levels and flow of ions across excitable membranes, among which KCC2 is an important component, may affect the pathophysiology of motoneuron disease.

Our findings provide evidence for an alternative mechanism of excitotoxicity that involves disinhibition of motoneurons. The KCC2 is a crucial factor for synaptic inhibition of motoneurons because the absence of KCC2 results in an excitatory action of GABA and glycine. Moreover, KCC2\textsuperscript{−/−} mice die immediately after birth because of severe motor deficits (22). In contrast to such a dramatic situation (and more relevant for most neurologic diseases), even a subtle downregulation of KCC2 may have profound consequences on the way intracellular Cl\textsuperscript{−} is handled by motoneurons; such perturbations may influence the strength and polarity of GABAergic and glycineric neurotransmission (32). As long as the Cl\textsuperscript{−} extrusion capacity of the motoneuron is kept high because of the normal expression and activity of KCC2, low intracellular Cl\textsuperscript{−} concentration is maintained. As a consequence, the reversal potential for GABA ($E_{GABA}$) is close to or even below the neurons resting membrane potential of approximately $\sim$63 mV (32). GABAergic and glycineric synaptic input then triggers Cl\textsuperscript{−} influx and hyperpolarization. On the contrary, a low Cl\textsuperscript{−} extrusion capacity due to a reduction in KCC2 expression and a subsequent change in $E_{GABA}$ may trigger Cl\textsuperscript{−} efflux and depolarization, thus increasing the excitability of motoneurons (33). In a rat model of spinal cord injury (17), a decrease of 10% to 20% in KCC2 protein content (similar to the results of this study) induced an approximately 10 mV depolarizing shift in $E_{GABA}$, mediated inhibitory postsynaptic potentials. Such a depolarizing shift might have a functional effect on GABA\textsubscript{A}, and glycine receptor responses (triggering action potentials instead of inhibiting them) depend on the degree of shift (33); the greater the upward shift in $E_{GABA}$, the more likely there will be a net excitatory action. Moreover, even small depolarizing inhibitory postsynaptic potentials may facilitate glutamatergic input, so that subthreshold excitations reach the action potential threshold (17). The limited effect of riluzole in the treatment of ALS and the prominent reductions in the numbers of glutamatergic synapses on spinal cord motoneurons during disease progression suggest that glutamatergic excitotoxicity may play only a minor role in the disease pathogenesis (34). Nevertheless, an interplay of pathways regulating and responding to changes in levels and flow of ions across excitable membranes, among which KCC2 is an important component, may affect the pathophysiology of motoneuron disease.
lead to an inactivation of KCC2, decreased plasmalemmal stability, and increased $[Cl^-]_i$. The extent to which these events occur during disease in SOD1-G93A mice and their significance are unclear. The observed redistribution of KCC2 away from the dendrites and the cell soma surface to the cytoplasm, however, suggests that such a functional impairment may be relevant in ALS.

Loss of motoneurons in ALS is accompanied by a robust inflammatory response including activation of microglia (39). Depending on their type and phenotypic activation states, microglia may exert either protective or toxic effects on motoneurons (40). In the initial neuroprotective state, microglia release neurotrophic factors such as brain-derived neurotrophic factor (BDNF), which are thought to help
motoneurons withstand cellular stress. In response to yet largely unknown signals, microglia switch their phenotype during disease and become cytotoxic instead. In this state, they release neurotoxic cytokines, nitric oxide, and oxygen radicals. The protective role of BDNF, however, is challenged by a study using acute hippocampal slices in which 100 ng/mL BDNF downregulated KCC2 mRNA and protein within 2 hours, resulting in a Cl\(^-\) exclusion capacity deficit (41). Likewise, in an animal model of chronic pain, microglia-derived BDNF elicited a depolarizing shift in the chloride gradient that made GABA excitable (42). Our observation of KCC2 downregulation in ALS-vulnerable motoneurons during disease progression suggests a potentially detrimental effect of BDNF signaling in the SOD1-G93A mouse model. Short-term changes in KCC2 transcription at presymptomatic stages recapitulating an “immature” molecular phenotype (43) may be part of a neuronal repair program, but when there is excessive glutamatergic neurotransmission in ALS, this may instead disturb Cl\(^-\) homeostasis, thereby promoting motoneuron damage and dysfunction.

Our behavioral assessment of the SOD1-G93A mice revealed a biphasic progression of tongue motor deficits: there was first a reduction in licking frequency at P77 and a second decline occurred at P126. On the molecular level, reductions in KCC2-ir first appeared at P80 in the hypoglossal subnucleus that innervates the genioglossal muscle, the tongue protruder. Loss of KCC2-ir subsequently spread over the entire nucleus and at P120 was even prominent in areas where still many motoneurons were present. Thus, the appearance of licking deficits and reductions in KCC2-ir occurred in parallel, suggesting that loss of KCC2 activity may be causally involved in the development of these motor disabilities.

During embryonic development and in adults in some mature neurons, NKCC1 functionally counteracts KCC2. Primarily, a high expression of NKCC1 in the rodent spinal cord is downregulated between embryonic day 17 and P20, with a concomitant shift from preferential white matter expression to gray matter expression (25). Our ISH analysis confirms this shift at the mRNA level with cellular resolution. Pathologic increases in NKCC1 expression and activity result in overexcitation of mature neurons in various neurologic diseases, including trauma, epilepsy, and neuropathic pain (8). The absence of an induction of NKCC1 mRNA expression in motoneurons during disease in SOD1-G93A mice compared to WT, as revealed by ISH in our study, however, suggests that this does not occur in this ALS model, at least for the motoneuron pools under study.

In summary, our molecular analyses of disease-related changes in KCC2 expression patterns suggest disturbed Cl\(^-\) homeostasis in vulnerable motoneurons in the SOD1-G93A mouse model of ALS. Further functional measurements are warranted to determine whether altered KCC2 expression effectively changes chloride homeostasis, for example, altering \(E_{\text{GABA}}\) or the chloride extrusion capacity. Moreover, the causes for the selective downregulation of KCC2 in ALS-vulnerable, but not in ALS-resistant motoneurons, require clarification. Strengthening neuronal inhibition through modulation of KCC2 expression and activity may ultimately represent a new therapeutic strategy for ALS.

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