Amyotrophic lateral sclerosis is a fatal, late-onset neurodegenerative disease, resulting from the progressive death of cortical and spinal motor neurons. About 90% of ALS cases are sporadic, and the remaining 10% are inherited in a dominant manner (familial ALS). The sporadic and familial forms of ALS are phenotypically indistinguishable suggesting a convergence of common pathogenic mechanisms in this disease. Insights into the pathological mechanisms underlying ALS came with the discovery of causative mutations in the enzyme Cu²⁺/Zn²⁺ superoxide dismutase (1). Transgenic expression of high levels of human SOD1 mutants (mutSOD1) in rodents leads to a progressive motor neuron disease that shares most of the clinical features of ALS (2, 3). Recent studies, performed in chimeric mice that are mixtures of normal and mutSOD1-expressing cells, have demonstrated that toxicity to motor neurons in the spinal cord requires damage from mutSOD1 acting in non-neuronal cells, including astrocytes (4). One of the prominent astrocyte functions is the regulation of glutamate concentration in the synaptic clefts of the central nervous system, not only to modulate the normal synaptic transmission but also to prevent the development of glutamate excitotoxicity and the consequent neuronal death. Up to 95% of the glutamate transport in the central nervous system is handled by the astroglial glutamate transporter EAAT2 (5, 6).

Several lines of evidence support a prominent role for EAAT2 impairment in the pathogenesis of ALS. Excitotoxicity caused by a consistent reduction in expression and activity of the glutamate transporter EAAT2 is among the various proposed pathogenic mechanisms implicated in playing a role in the propagation of disease (7). In the G93A-SOD1 transgenic mouse and rat, the expression levels of EAAT2 are reduced, often focally in the spinal cord ventral gray matter and prior to the onset of the disease (3). EAAT2 knockdown achieved by using antisense oligonucleotides in adult rats leads to hind limb paralysis with motor neuron degeneration and denervation similar to ALS (8). We have reported previously that mutSOD1 proteins, heterologously expressed in Xenopus oocytes exposed to oxidative stress, lead to selective inhibition of EAAT2 by targeting the C-terminal domain (9). Moreover, experiments in which EAAT2 was genetically or pharmacologically increased have led to the conclusion that EAAT2 impairment contributes to the progression of motor neuron degeneration in ALS (10–12).

Several experimental observations indicate that mutSOD1 toxicity involves sequential activation of caspase-1 and caspase-3 in transgenic mice and in cellular models of ALS (13–15). Of particular interest is the role that caspase-3, a key proteolytic enzyme executioner of apoptosis (16), plays in ALS. Oxidative stress delivered to the N2a cell model of mutSOD1-linked ALS triggered caspase-3 activation (17). Evidence for activation of caspase-3 was also reported in vivo in mutSOD1 transgenic mice, both in motor neurons and astrocytes of the spinal cord at the time of onset of ALS-like symptoms (13). In addition, modulation of caspase-3 activity by overexpression of Bcl-2 or by infusion of the caspase inhibitor Z-VAD-fmk provided protective benefits in a transgenic mouse model of ALS (18–20). Although it is not clear whether activation of caspase-3 initiates ALS, the observations accumulated thus far strongly indicate that this mechanism contributes substantially to the progression of the disease.

EAAT2 has a putative consensus site for caspase-3 cleavage located in the cytoplasmic C-terminal domain. In this study, we provide evidence
that the C-terminal domain of EAAT2 is cleaved by caspase-3 and that this proteolytic process functionally impairs the transporter activity. Cleavage and inactivation of EAAT2 are triggered by mutSOD1 when challenged by oxidative stress \textit{in vitro}. Moreover, we found evidence of a truncated EAAT2 form likely derived from caspase-3 cleavage in the spinal cord of G93A-SOD1 transgenic ALS mice, suggesting that this event also occurs \textit{in vivo}.

**EXPERIMENTAL PROCEDURES**

**Animals**—Wild type, G93A-SOD1 transgenic mice, and nontransgenic control mice were used for the study. The mouse colonies were B6SJL-TgN (G93A-SOD1)1Gur (stock number 002726; \sim 30 copies of the transgene; The Jackson Laboratory, Bar Harbor, ME), and B6SJL-TgN(SOD1)2Gur (stock number 002297; \sim 8 copies of the transgene) and were maintained in-house. These lines expressed approximately the same amount of human SOD1 protein as assessed on immunoblot. Genotyping and determination of transgene copies number were performed by PCR analysis.

**Transfections and Protein Extractions**—HEK 293 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. EAAT2 and D505N-EAAT2 cloned in pcDNA3.1 were transiently transfected in HEK 293 cells at \sim 70% confluence using Lipofectamine 2000 (Invitrogen). Transfected cells were harvested 48 h later in buffer containing CHAPS (1%), 150 mM NaCl, 10 mM NaPi, pH 7.4, and Complete\textsuperscript{TM} protease inhibitor mixture (Roche Applied Bioscience), briefly sonicated, and stored at \sim 80 °C for Western blot analysis or immediately processed for caspase cleavage reactions.

**In Vitro Cleavage Reactions**—Mice were euthanized by intraperitoneal injection of xylazine/ketamine according to institutional guidelines. Spinal cords were collected and immediately homogenized on ice (glass-Teflon homogenizer; 1,000 rpm) in 30 volumes of hypotonic solution containing 2 mM EDTA, 10 mM NaPi, pH 7.4, and protease inhibitors (Complete Mini\textsuperscript{TM} with EDTA). The homogenates were centrifuged (39,000 \times g, 15 min), supernatants removed, and the “crude” membrane pellet resuspended in extraction buffer containing SDS or CHAPS (1%), 150 mM NaCl, 10 mM NaPi, pH 7.4, and Complete\textsuperscript{TM} protease inhibitor mixture (Roche Applied Bioscience), briefly sonicated, and stored at \sim 80 °C for Western blot analysis or immediately processed for caspase cleavage reactions.

**Glutamate Transporter Mutations**

**Site-directed Mutagenesis and PCRs**—D505N-EAAT2 was generated using the QuikChange\textsuperscript{TM} site-directed mutagenesis kit (Stratagene, La Jolla, CA), confirmed by sequencing and cloned in pcDNA3.1 for mammalian expression or pOX for oocyte expression. Asparagine was chosen rather than the more commonly used alanine because the structural similarity of asparagine to aspartate did not alter the activity and trafficking of D505N-EAAT2 in oocytes, which was a crucial requisite to assess the impact of the caspase-3 treatment on the transporter function. The coding region of the truncated EAAT2 (Tr)EAAT2 was generated by PCR and the cDNAs subcloned in pcDNA3.1.

**Antibodies**—Affinity-purified polyclonal antibodies against peptides of the glutamate transporters EAAT1–3 were referred to by capital letters A, B, and C, respectively, followed by numbers indicating the corresponding peptide of the rat transporter sequence. For this study we used A522-541 (0.2 \mu g/ml, rabbit 8D0161, EAAT1 cytoplasmic C terminus), B12-26 (0.2 \mu g/ml, rabbit 26970, EAAT2 cytoplasmic N terminus), B493-508 (0.1 \mu g/ml, rabbit 84946, EAAT2 cytoplasmic C terminus), and C161-177 (0.5 mg/ml, EAAT3 extracellular loop, Zymed Laboratories Inc.). Moreover, an antibody raised against the last 17 amino acids of EAAT2 C terminus was purchased from Affinity BioReagent (which we termed ABR556-573, catalogue number PA3-040; 1:1,000–10,000). These anti-glutamate transporter antibodies, although raised against the rat sequence, cross-react with mouse and human isoforms except for B518-536, which does not cross-react with the mouse isoform. Other antibodies used are anti-human SOD1 (Calbiochem) and anti-p17 fragment of caspase-3 (Cell Signaling).

**Protein Expression in Oocytes**—Linearized plasmids were transcribed \textit{in vitro} using the mMessage mMachine kit (Ambion, Austin, TX). Stage V \textit{Xenopus} oocytes were enzymatically defolliculated, injected with cRNAs (20–50 ng/oocyte), incubated at 18 °C in L-15 solution (Specialty Media) supplemented with gentamycin sulfate (100 \mu g/ml) and used for experiments 1–3 days post-injection.

**Glutamate Uptake Measurements**—Glutamate uptake current and uptake of \textit{l-[3H]}glutamate were measured in oocytes as described previously (9). To assess the effect of caspases on glutamate-evoked EAAT2 uptake current, 0.2–4 ng of active recombinant purified caspases (BD Biosciences) in 50 \mu l of buffer were injected into the oocytes, and the uptake current was recorded before and after the injection. Injection buffer contained (final concentration in the oocyte in mM) the following: 1 Tris-HCl, 2 NaCl, 1 imidazole, 10 KPi, pH 7.4, 0.01% CHAPS, 0.1 EDTA, 1 DTT, and 0.2% glycerol. Glutamate uptake was measured for 5 min by incubating the oocytes with 10 \mu M glutamate isotope-diluted with \textit{l-[3H]}glutamate (specific activity 51.9 Ci/mmol; PerkinElmer Life Sciences) in 1 ml of frog Ringer solution (115 mM NaCl, 2 mM KCl, 1.8 mM CaCl\textsubscript{2}, 10 mM HEPES, pH 7.1–7.3). The uptake reaction was stopped by rinsing the oocytes with cold sodium-free frog Ringer solution (0 Na\textsuperscript{+}, 115 mM choline). The oocytes were then dissolved in 10% SDS and counted to quantify the incorporation of glutamate.

**Caspase Activity Assay**—Ten oocytes/group were incubated for 1 h with H\textsubscript{2}O\textsubscript{2} (150 \mu M). Each group was lysed in 400 \mu l of buffer containing 50 mM HEPES, pH 7.4, 0.1% CHAPS, 1 mM EDTA, 1 DTT, and incubated for 5 min in an ice bath. The lysate was centrifuged at 16,000 \times g (15 min at 4 °C), and 5 \mu l of supernatant was used to determine caspase-3 cleavage activity and kinetics (>12 h) using a fluorometric assay (BD Biosciences). Fluorescence emission was determined by a Fluor-star BMG fluorometer (excitation 380 nm and emission 420 nm). \textit{l-[3H]}Glutamate uptake was measured for 5 min in oocytes treated for 1 h with 150 \mu M H\textsubscript{2}O\textsubscript{2} or control as described in the legend to Fig. 6.

**Biotinylation of Cell Surface Transporters**—Biotinylation reactions were performed using the biotinylation kit from Pierce and following the manufac-

**Caspase-3-mediated Processing of EAAT2**

Read the rest of the document for further details and methods.
manufacturer’s protocol with minor adaptations for Xenopus oocytes. Briefly, 20–25 oocytes/group were used for these experiments. The high number of oocytes per group was intended to minimize intra-group variability because of unpredictable variations in the expression levels of EAAT2 molecules targeted at the cell surface of the oocyte.

RESULTS

Caspase-3 Cleaves the Cytoplasmic C-terminal Domain of EAAT2 at Aspartate 505 — The cytoplasmic C-terminal domain of the glutamate transporter EAAT2 has a unique putative caspase-3 cleavage motif located at aspartate 505 (human EAAT2 sequence numbering). This aspartate (Asp-505) is conserved among isoforms from different species, including rat and mouse (Fig. 1A). To test whether EAAT2 could be cleaved by caspase-3, we treated mouse spinal cord homogenates with increasing amounts of purified caspase-3 (active form of the recombinant human caspase-3, concentration range 10–100 nM), and the reaction was then probed on Western blot with three polyclonal anti-EAAT2 antibodies directed against different domains of the transporter (Fig. 1A). Caspase-3 concentrations used in this experiment are comparable with those of other studies reporting caspase-3 cleavage of different substrates, such as PARP or amyloid precursor protein (21). The treatment led to a dose-dependent loss of EAAT2 immunoreactivity and a simultaneous dose-dependent appearance of a lower band that corresponded to the shortened form of EAAT2 (Fig. 1B, (Tr)EAAT2) when the blot was probed with the anti-EAAT2 N-terminal antibody B12-26. The same reaction probed with the antibodies B493-508 (Fig. 1B) and ABR556-573 (Fig. 1B) showed dose-dependent loss of EAAT2 immunoreactivity and no evidence for (Tr)EAAT2 accumulation, as expected if the cleavage occurred at the unique site at aspartate 505. Failure to recognize (Tr)EAAT2 by the B493-508 antibody, whose epitope sequence encompasses the putative caspase-3 consensus site in EAAT2, argues that the immunoreactivity of this antibody is mainly directed against the last amino acids of the C-terminal portion of the epitope. This is also supported by evidence that B493-508 immunoreactivity toward EAAT2 is abolished by incubating the antibody with the peptide 506–516 (100 μM, human EAAT2 sequence numbering), whereas the peptide 493–505, although it slightly decreased the affinity of the antibody for EAAT2, it did not block the antibody immunoreactivity toward EAAT2.
The cleavage of EAAT2 is specific for caspase-3 as other caspases, such as caspase-1, -6, -7, and -8, were ineffective (Fig. 1C). EAAT2 translation with [35S]methionine was incubated with active caspase-3, and the reaction was resolved on 10% gel and probed with the B12-26 antibody (diluted 1:1,000). 5 μg of in vitro translated wild type EAAT2 and D505N mutant (B), EAAT1 (C), and EAAT3 labeled with [35S]methionine (D) were treated for 1 h at 37 °C with 1 μl of active caspase-3 (Casp-3), resolved on 12% gels, and developed by autoradiography. CTE is the abbreviation for the C-terminal domain of EAAT2 and is the fragment downstream (from the N to the C terminus) from the caspase-3 cleavage site. Because of the lack of proper glycosylation mechanisms in the translated in vitro system, in vitro translated EAAT2 runs at ~60 kDa, which is ~15 kDa lower than native EAAT2. The time course for caspase-3 cleavage of the in vitro translated EAAT2 is much faster than that of EAAT2 in tissue homogenates. At 1 h, ~30% of EAAT2 is cleaved in the tissue homogenate versus the totality of the in vitro translated EAAT2, likely reflecting the fact that in tissue homogenates caspase-3 is consumed by other potential substrates.

To ascertain whether the cleavage occurred at the predicted site in the EAAT2 sequence, we disrupted the putative consensus site in EAAT2 by mutating aspartate (D) at position 505 to asparagine (N). CHAPS extracts of HEK 293 cells transfected with wild type and D505N-EAAT2 mutant were then treated with caspase-3 and analyzed by immunoblot. As expected, the D505N mutation prevented the cleavage from occurring (Fig. 2A). We also tested whether other unconventional sites could possibly be used in addition to the canonical one. In vitro translated EAAT2 labeled with [35S]methionine was incubated with caspase-3 and the reaction analyzed by autoradiography. EAAT2 has 24 methionine residues evenly scattered in the primary structure, including 2 methionines located downstream of the caspase-3 consensus motif, ensuring even labeling of all domains of the transporter. Caspase-3 cleaved EAAT2 but not D505N-EAAT2, forming two fragments at ~55 kDa ((Tr)EAAT2) and 8 kDa (termed CTE as for the C-terminal domain of EAAT2 excised by caspase-3) (Fig. 2B). It follows that the canonical motif for caspase-3 cleavage is the only site used to cleave EAAT2.

EAAT1, the other major astroglial glutamate transporter, lacks conventional cleavage sites for caspase-3 and was indeed insensitive to caspase-3 treatment (Fig. 2C and see also Fig. 4E). On the contrary, the neuronal glutamate transporter EAAT3 has two caspase-3 cleavage sites defined by the sequence -DXXD-, also located at the cytoplasmic C terminus. Cleavage at these sites would generate small fragments of ≤3 kDa, which is hard to detect on the gel. Moreover, these fragments do not contain methionines and therefore could not be visualized by autoradiography. Indeed, treatment of the in vitro translated EAAT3 with caspase-3 did not produce proteolytic fragments. Nevertheless, we detected a small but reproducible downward shift of the EAAT3 band, suggesting that the cleavage may have occurred (Fig. 2D and see also Fig. 4C).

**EAAT2 Cleavage by Caspase-3 Leads to Uptake Inhibition—**Despite EAAT2 being an astroglial protein, previous studies have shown that primary astrocytes in culture do not express physiological levels of EAAT2. *In vivo* manipulation of astrocyte cultures with cell differentiation-inducing compounds, such as cAMP analogues or epidermal growth factor, promoted expression of EAAT2 (22). However, these manipulations resulted in expression of non-functional transporters, raising the concern that EAAT2 was not properly targeted to the plasma membrane or was incorrectly processed in this system, therefore questioning the validity of the astrocytes as an experimental model system to study the function of EAAT2 (23, 24). To assess whether caspase-3 proteolytic cleavage had a functional impact on EAAT2, we therefore took advantage of the oocyte expression system and the two-electrode voltage clamp technique to measure EAAT2 uptake current. *Xenopus* oocytes injected with cRNA for EAAT2 abundantly express a functional transporter (25). EAAT2-expressing oocytes were then injected with the active caspase-3, and EAAT2 uptake current was measured before and after the injection. At ~50 mV, EAAT2 uptake current was rapidly and progressively inhibited by the injection of caspase-3 (~45% in 20 min), whereas the uptake current of the D505N-EAAT2 mutant was unaltered, suggesting that the proteolytic cleavage at the caspase-3 consensus site in EAAT2 was responsible for the inhibition (Fig. 3, A and B). Affinity for substrate (i.e. $K_m$ for glutamate) calculated from the glutamate-evoked current of the D505N-EAAT2 mutant transporter expressed in oocytes was statistically not different from wild type EAAT2 (20.2 ± 4.5 μM for WT-EAAT2 versus 17.7 ± 5 μM for D505N-EAAT2, $n$ = 3 oocytes; data not shown); in addition, no change in glutamate uptake velocity was also observed (23 ± 4 for WT-EAAT2 versus 25 ± 3 nmol/mg protein/15 min for D505N-EAAT2, $n$ = 10 oocytes/group, measured at 10 μM glutamate; see “Experimental Procedures”). A similar uptake activity between wild type and D505N-EAAT2 is consistent with a lack of significant structural alterations in the mutant D505N-EAAT2. The transport of glutamate is coupled to the movements of cations across the plasma membrane (Na, K, and H) that are necessary for transporter cycling and glutamate uptake, as well as chloride anions that are uncoupled to the flux of glutamate. The relative proportion of the uptake current generated by ion-coupled glutamate transport (stoichiometric current) and the glutamate-gated chloride conductance varies in intensity among EAAT isoforms (26). In EAAT2, the stoichiometric current is predominant compared with the chloride conductance (26). However, to rule out that the caspase-3-mediated inhibition of the uptake current was because of alterations in the glutamate-gated chloride...
current component rather than the transport of glutamate, we measured the effect of caspase-3 on EAAT2 uptake current at the chloride equilibrium potential (−20 mV), whereby the contribution of this conductance to the total uptake current is nullified. The extent of inhibition (45% at 20 min at −20 mV; data not shown) was the same as at −50 mV, suggesting that the glutamate transport current was the EAAT2 uptake current component affected by the caspase-3 cleavage. EAAT2 inhibition increased at increasing amounts of injected caspase-3 (Fig. 3C). Other caspases like caspase-7 (Fig. 3A) or caspase-1 and -6 (not shown) did not affect EAAT2, an observation in accord with the lack of effect of these caspases on EAAT2 in the in vitro cleavage reaction. Despite the fact that caspase-8 did not directly cleave EAAT2 in vitro (Fig. 1C), when injected into oocytes expressing EAAT2, it caused a progressive inhibition of the transporter-mediated uptake current, although with much slower kinetics because 50% inhibition was achieved in 60 min post-injection compared with 20 min for caspase-3 (not shown). Because caspase-8 is an upstream regulator of the caspase cascade (27) and its inhibitory effect on EAAT2 was prevented by the caspase-3 inhibitor Z-DEVD-fmk (50 μM in 0.1% Me2SO; not shown), it is likely that caspase-8 inhibition was mediated by activation of the endogenous pool of caspase-3. Caspase-3 also inhibited the uptake of radiolabeled glutamate in a time-dependent fashion by intracellular injection of active caspase-3. Oocytes were injected with 2 ng of active caspase-3 or with vehicle (n = 10), and at the indicated times, uptake of [3H]glutamate was measured for 5 min.

FIGURE 3. Caspase-3 cleavage of EAAT2 inhibits its activity. A, representative recordings obtained from oocytes expressing wild type or D505N-EAAT2 mutant. Whole-cell currents were recorded in gap-free mode at −50 mV; glutamate-elicited uptake currents (100 μM l-glutamate; filled bars) were measured before and after every 5 min after injection of caspase-3 (Casp-3) (2 ng/50 nl) or vehicle (arrow indicates the time of injection during the recording). Recordings were made from oocytes bathed in Oocyte Ringer solution containing (in mM) 115 NaCl, 2 KCl, 1.8 CaCl2·2H2O, 10 HEPES, pH 7.1–7.3. Microelectrodes were filled with 3 M KCl and had a tip resistance of 0.5–2 megohms. The effect of caspase-3 on EAAT2 activity was determined by comparing the peak EAAT2 currents before and after the injection points. Changes in uptake currents as in A were plotted from data obtained from at least 8 oocytes/group. B, caspase-3 inhibition on EAAT2 activity is dose-dependent. EAAT2 uptake currents were measured at 20 min post-injection of increasing concentrations of injected caspase-3. C, EAAT2-mediated uptake of [3H]glutamate is inhibited in a time-dependent fashion by intracellular injection of active caspase-3. Oocytes were injected with 2 ng of active caspase-3 or with vehicle (n = 10), and at the indicated times, uptake of [3H]glutamate was measured for 5 min.

FIGURE 4. The glial transporter EAAT1 and the neuronal transporter EAAT3 are insensitive to caspase-3. A, oocytes expressing EAAT3 are injected with 2 ng of active caspase-3 during recording at the time indicated by the arrow. The transporter-evoked uptake currents were elicited by application of 100 μM glutamate (solid boxes). Numbers represent % of uptake current (100% is the current before injection) and are the average ± S.E. of at least three different recordings. B, [3H]glutamate uptake was measured for 5 min after 15 or 25 min post-injection of active caspase-3. C, Western blot analysis of a nontransgenic mouse spinal cord homogenate (CHAPS extract) treated with increasing concentration of caspase-3. D, oocytes expressing EAAT1 were injected with 2 ng of active caspase-3 (arrow), and uptake currents were evoked by application of 100 μM glutamate (n = at least 5). E, mouse spinal cord CHAPS extract treated as in C and analyzed on Western blot with the anti-A522-541 antibody for the glutamate transporter EAAT1.
min at room temperature with [3H]glutamate as substrate. Uptake activity compared with wild type EAAT2. Glutamate uptake was measured for 5 min, which is still present as monomer at (Tr)EAAT2 has a lower tendency to aggregate in a dimeric or trimeric form compared with EAAT2 assessed as the biotinylatable fraction. Note that (Tr)EAAT2 is comparable with EAAT2 assessed by Western blot of the oocytes analyzed on Western blot to assess the expression of human SOD1 with an antibody that recognizes both human and Xenopus SOD1 (Calbiochem). Human SOD1 could be detected because of its slightly higher mobility on the gel. Human SOD1 expression levels were comparable in the A4V and WT groups. Oocytes expressing wild type EAAT2 as well as a large portion is still present as monomer at ~75 kDa on Western blot. C, despite comparable cell surface expression, (Tr)EAAT2 has ~65% less uptake activity compared with wild type EAAT2. Glutamate uptake was measured for 5 min at room temperature with [3H]glutamate as substrate.

Although EAAT2 was functionally impaired by caspase-3, this was not accompanied by retrieval of the transporter from the plasma membrane. Oocytes did not observe a decrease in biotinylatable EAAT2 during the time frame of the experiment in parallel to the inhibition of EAAT2 uptake current (Fig. 5A). However, we cannot rule out that in the long term the truncated transporter is retrieved from the plasma membrane to be degraded. To ascertain whether the cleaved transporter, although still at the plasma membrane, has impaired activity, we expressed a PCR-generated (Tr)EAAT2 in oocytes, and we compared its uptake activity with wild type EAAT2. On average, the PCR-generated (Tr)EAAT2 showed ~65% less L-[3H]glutamate uptake activity than wild type EAAT2, even though the two transporter forms appeared to have similar expression levels at the plasma membrane (Fig. 5, B and C).

**Familial ALS-linked Mutant SOD1 Proteins Inhibit EAAT2 Largely via Activation of Caspase-3**—We have shown previously that in the presence of oxidative stress, mutSOD1 triggers inhibition of EAAT2 by targeting the cytoplasmic C-terminal domain of the transporter (9). Furthermore, when N2a cells were challenged with oxidative stress, the expression of mutSOD1 led to increased caspase-3 cleavage activity (17), suggesting a link between oxidative stress, mutSOD1 toxicity, and caspase-3 activation. We wanted to determine whether and to what extent mutSOD1 inhibition of EAAT2 involved caspase-3 activation and cleavage. In our previous work, we characterized the inhibitory effect of A4V-SOD1 mutant (the most common SOD1 mutation in familial ALS patients in North America (28)) on EAAT2 expressed in Xenopus oocytes coexpressing wild type EAAT2 (T2), D505N mutant (T2-D505N), and different SOD1 mutants (10 oocytes/experimental group) were incubated for 1 h in 150 μM H2O2 in oocyte Ringer solution containing (in mM) 115 NaCl, 2 KCl, 1.8 CaCl2·2H2O, 10 HEPES, pH 7.1–7.3, and in the presence or absence of the specific caspase-3 inhibitor Z-DEVD-fmk (50 μM in 0.1% Me2SO). Oocytes were preincubated 30 min with Z-DEVD-fmk before exposure to H2O2.

Uptake of L-[3H]glutamate was measured for 5 min at room temperature and compared among groups of oocytes treated with H2O2 ± Z-DEVD-fmk and treated with 0.1% Me2SO as control. Data were expressed as percentage of inhibition versus control-treated oocytes. Expression levels of SOD1 mutants were determined by Western blot on aliquots of oocyte homogenates and found identical among different groups. Data were obtained from at least five different experiments and are expressed as mean ± S.E.
and EAAT2 + A4V-SOD1, respectively. In these experiments, expression levels of WT and A4V-SOD1 were equivalent as determined on immunoblot (Fig. 6B). Similarly to A4V-SOD1, caspase-3 activation was also achieved with the G93A and H46R-SOD1 mutations (data not shown) from which (29) transgenic mice and rat models of ALS have been generated (29). In parallel to the mutSOD1-dependent caspase-3 activation, we also measured a consistent decrease in EAAT2-mediated [3H]glutamate uptake activity (≥40%). This inhibition was largely blocked (≥60%) by pretreatment with the caspase-3 blocker, Z-DEVD-fmk, suggesting a direct involvement of caspase-3 in the inactivation of EAAT2. The extent of mutSOD1-mediated inhibition of D505N-EAAT2 was similar to the mutSOD1-mediated inhibition of EAAT2 in the presence of Z-DEVD-fmk, suggesting that caspase-3 cleavage in the cytoplasmic C-terminal domain of EAAT2 was largely, although not entirely, responsible for the inhibition (Fig. 6C).

Evidence for Caspase-3-mediated EAAT2 Cleavage in the Spinal Cord of G93A-SOD1 Mice Model of ALS—Caspase-3 activation (13), apoptotic cell death (15), and impairment in expression and activity of the glutamate transporter EAAT2 (30) are pathological events occurring in both human ALS and transgenic models of the disease. We wanted to determine whether EAAT2 could also be a substrate for caspase-3 cleavage in vivo in an animal model of ALS, in an effort to link activation of caspas and excitotoxicity as convergent mechanisms in the pathogenesis of ALS. We looked for the presence of the proteolytic fragment (Tr)EAAT2 suggestive of EAAT2 processing by caspase-3 in spinal cord homogenates of G93A-SOD1 mice at different stages of disease progression using the B12-26 antibody directed against the N terminus of EAAT2. We also looked for loss of EAAT2 immunoreactivity using the B493-508 antibody directed against the epitope that encompasses the caspase-3 cleavage motif in EAAT2. A pronounced and progressive loss of EAAT2 immunoreactivity was detected with the antibodies B12-26 and B493-508. On average, EAAT2 immunoreactivity probed with B12-26 and B493-508 at disease end stage decreased by 47 ± 4% (n = 6, S.E.) (Fig. 7F). The decrease was significant already (12 ± 7%, n = 6, S.E.) at the pre-symptomatic stage (day 80; Fig. 7, A, D, and F). At brief exposure (<2 min) of the Western blot, the B12-26 antibody probed a consistent decrease in immunoreactivity for EAAT2 (Fig. 7A) but no other proteolytic EAAT2 fragments. However, a prolonged exposure of the blot (>30 min) revealed an immunopositive band at a slightly lower molecular weight that likely corresponds to (Tr)EAAT2 and could be detected already at a pre-symptomatic stage and more pronounced at end stage. Furthermore, the band was not detected either by the B12-26 antibody of EAAT2 both at brief (<2 min) and at much longer exposures (>30 min; Fig. 7D) or by the ABR556-573 antibody directed against the C terminus of EAAT2 downstream from the caspase-3 consensus site (not shown). The presence of (Tr)EAAT2 is strongly suggestive of caspase-3 cleavage occurring during the course of the disease in these mice. Along with its appearance, we observed a progressive accumulation in the p17 fragment of caspase-3 in the spinal cord of these ALS mice occurring at disease progression (Fig. 7C), indicating increased caspase-3 activation.

Discussion

Several lines of evidence suggest a crucial role for the impairment of the EAAT2 transporter in the propagation of motor neuron death in ALS (31–33). Mechanisms accounting for impairment of EAAT2 expression levels and activity may include altered transcription or splicing, post-translational modifications, decreased synthesis, accelerated degradation-processing rates, and impaired targeted trafficking to and from the plasma membrane, in essence, all events that ultimately reduce cellular glutamate transport capacity and lead to disruption of the glutamate homeostasis in the central nervous system. Our data indicate that caspase-3-mediated proteolytic cleavage of EAAT2 is an additional mechanism to selectively inhibit this glutamate transporter. Activation of caspase-3 and the specific cleavage reactions associated with it are a central event in the cell death mediated by mutant SOD1 in ALS. Because EAAT2 has a predominant astrocytic localization, activation of caspase-3 is anticipated to be in the astrocytes for the cleavage to occur. Indeed, activation of caspase-3 was reported both in motor neurons and astrocytes at the time of the earliest motor neuron death in three different mouse models of ALS (13, 14, 18). Caspase-3 activation and cleavage of EAAT2 are not necessarily linked to the demise of the cells in which this event occurred. There is no evidence indicating that spinal astrocytes in ALS undergo apoptotic cell death. Apoptosis can also occur in the absence of caspase activation (34), whereas caspase activation does not systematically mediate cell death. Several lines of evidence have shown that constitutive, non-lethal activation of caspase-3 can occur

![Figure 7](image_url)

**FIGURE 7. Evidence for caspase-3-mediated cleavage of EAAT2 in the spinal cord of G93A-SOD1 transgenic mice during ALS progression.** A and B, representative Western blots of spinal cord homogenates (SDS extracts) of G93A-SOD1 mice at different stages of disease progression (p.s., pre-symptomatic stage, 70 days old; e.o., early at onset of symptoms, 100 days old; e.s., end-stage, ~130 days old) were probed with the N-terminal EAAT2 antibody B12-26. Spinal cord homogenates from human WT-SOD1-overexpressing mice, age-matched with end-stage G93A-SOD1 mice, were used as control. A and B represent short (<2 min) and longer (>30 min) exposure of the blots. C, the homogenates were also probed with an antibody that recognizes the p17 fragment of active caspase-3. D and E, the same SDS extracts were also probed with the antibody B493-508, which recognizes the epitope that encompasses the putative caspase-3 cleavage site in EAAT2. D and E represent short (<2 min) and longer (>30 min) exposure of the blots. F, loss of EAAT2 immunoreactivity was quantified by densitometric analysis using ImageJ version 1.30 by averaging the densitometric values determined with the anti-EAAT2 antibodies B12-26 and B493-508 (upper panel); *p < 0.05). Values are mean ± S.E. of at least six experiments.
and may be responsible for proteolytic processing of a variety of proteins within the cell, with potentially toxic consequences or with normal physiological and beneficial implications. Recent studies have highlighted a role of activated caspases in nonapoptotic pathways, including inflammatory responses, immune cell proliferation, differentiation of various cell types, and others (35, 36). The fine regulation of these events remains poorly understood. In this context, EAAT2 cleavage should not be simply seen as a nonspecific event, even in an apoptotic setting whereby caspase-3 has been activated by pathological factors. In fact, the evidence that there are non-apoptotic or non-lethal functions of caspases implicates that cells have the ability to control and restrict the proteolytic activity of these enzymes to selected substrates (37) in order to avoid unspecified dismantling of the cell. Various mechanisms could account for these specific and selective cleavage reactions, including post-translational modifications of caspases and/or substrates, subcellular compartmentalization of caspases, protection of potential target proteins by scaffold molecules, and compartmentalized activation of antiapoptotic factors.

Analysis of the caspase-3 cleavage kinetics indicates that EAAT2 is cleaved with comparable affinity to well described substrates for caspase-3 such as PARP or amyloid precursor protein, suggesting that the EAAT2 cleavage is a cell-relevant processing event. Functionally, caspase-3-mediated EAAT2 cleavage drastically impaired EAAT2 activity. The wild type transporter normally assembles in the plasma membrane as a homotrimer (38). The cleavage initiated by caspase-3 does not cause immediate internalization of the truncated transporter, suggesting that the impairment in activity observed during the initial phase of caspase-3 cleavage is most likely due to the resulting derangement of the native quaternary structure and loss of integrity of the transporter trimeric assembly. In line with this, we observed that despite normal forward trafficking of the truncated EAAT2 to the cell surface, its activity is dramatically compromised. Because the caspase-3 cleavage site is in close proximity of EAAT2 transmembrane domain 8 (TM8) (38), which appears to be relevant for the binding and transport of glutamate, the removal of the majority of the cytosolic C-terminal domain by caspase-3 cleavage could affect the normal conformational arrangement of TM8 in the EAAT2 architecture, thereby altering negatively the transport kinetics.

We reported previously that mutant SOD1 proteins linked to familial ALS cause functional inactivation of EAAT2 in vitro by targeting the C-terminal domain (9). We have now determined that about 60% of that inhibition is caused by caspase-3-mediated cleavage of the C-terminal domain of EAAT2. A residual 40% of the total inhibition is still not dependent upon caspase-3 cleavage, suggesting that the aberrant activity of mutSOD1 targets EAAT2 by possibly acting on multiple direct or indirect molecular pathways. Further studies are required to characterize these additional mutSOD1-mediated inhibitory mechanisms.

How mutSOD1 activates caspase-3 in ALS remains poorly understood. Nevertheless, it appears that the sequelae of events that are initiated by mutSOD1 and lead to activation of caspase-3 in astrocytes ultimately impair EAAT2. Decreased expression and activity of EAAT2 in ALS cannot be attributed simply to cell death because no significant loss of astrocytes was reported in the disease. Furthermore, it seems that the expression levels of the other major glial transporter, EAAT1, are preserved throughout disease progression. This evidence is in accord with our observation that EAAT1 is functionally and proteolytically insensitive to caspase-3. Instead, a chronic and sustained increase in caspase-3-mediated proteolytic processing of EAAT2 may unbalance the turnover rate of the transporter in favor of its degradation pathways and therefore account, at least in part, for the EAAT2 loss in ALS. In many cases, the functional consequences of caspase-3 cleavage of a target protein on subsequent apoptotic or nonapoptotic events are not clear. However, in a number of cases cleavage has been clearly shown to inactivate proteins that normally contribute to promote cellular survival or to convert normally benign polypeptide or enzymes into pro-apoptotic molecules (16). Activation of caspase-3 in astrocytes, although it may not lead to the astrocyte death, even in a disease setting like in ALS, could result in the overall loss of glutamate transport capacity and accumulation of extrasynaptic glutamate because of the selective cleavage of EAAT2.

The mechanisms underlying the pathogenesis of selective motor neuron death in ALS are not completely understood and may involve many different factors perhaps converging from many different cell types of the spinal cord or muscle cells. Our study reported the novel evidence that the glutamate transporter EAAT2 could undergo proteolytic cleavage by caspase-3. This functionally impairs glutamate transport activity and could ultimately contribute to the neuronal death observed in ALS.

Although EAAT2 loss is not regarded as initiator of the disease, mounting lines of evidence point to a role for EAAT2 down-regulation as an important contributor to the pathogenesis of ALS that could act to propagate motor neuron degeneration in this disease.

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